UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

Dipartimento di Agraria

Final dissertation for the achievement of the degree of

Doctor (Ph.D.)

in

Scienze agrarie e agroalimentari / Agricultural and food sciences

XXX ciclo

The use of wood anatomical traits such as Intra-Annual Density Fluctuations as a tool to gain insights into Mediterranean plant adaptation to drought

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Academic year 2017/2018

INDEX

1. INTRODUCTION

1.1 Overview

The influence of intra-seasonal environmental factors on tree growth has been increasingly investigated with the analysis of intra-annual tree-ring wood anatomical traits which provides significant information for ecological and climatological studies especially when combined with dendrochronological investigations and eco-physiological information deriving from the analysis of carbon and oxygen stable isotopes in tree-ring series. Understanding the relationships between wood traits and environmental factors is useful to reconstruct past reactions of trees to climate and helps predicting tree species responses to forecasted climate changes; this would also help evaluating the potential forest vulnerability to peculiar intra-annual climate stresses such as drought (McDowell et al., 2008).

Mediterranean ecosystems represent a newsworthy study area for the investigation of plant intra-seasonal responses to climate, where precipitation and temperature fluctuations lead to the succession of different periods of water deficit and surplus during the year, affecting wood growth (Cherubini et al., 2003). Moreover, Mediterranean area is considered to be one of the climate change hotspots, being a transition zone between temperate and tropical regions (Diffenbaugh and Giorgi, 2012). It has experienced an increase in drought frequency in the past three decades, and forecasts predict even higher irregularities in the intra-annual precipitation patterns and increasing temperature in the next ones (IPCC, 2017). In Mediterranean ecosystems, tree species are adapted to cope with the so-called “double stress” due to summer drought and winter low temperatures (Cherubini et al., 2003), thanks to a high plasticity in wood functional traits which allows them to preserve hydraulic conductivity in the different seasons achieving a trade-off between hydraulic efficiency and safety (Beeckman, 2016). Plastic adjustments can be observed in wood showing peculiar structural traits which are differently modulated according to species, sites or to the combination of both (Fonti et al., 2010). Climate variability can therefore be recorded in tree-ring plastic adjustments of wood density which depends on conduit lumen area and cell wall thickness;

in hardwoods, other traits such as vessel frequency can be considered. Intra-annual variability of anatomical traits result in changes in wood density which is considered an indicator of photosynthate availability. Wood functional trait adjustments due to Mediterranean double stress are the result of a bimodal growth pattern resulting from peculiar cambial activity and cell differentiation phases (Schweingruber, 2007). Such xylogenesis pattern lead to the formation of the so-called "intra-annual density fluctuations" (IADFs) in tree rings. IADFs have been often referred to as false rings and double rings in Mediterranean woods (De Micco et al., 2016). The characterization of IADFs in tree rings, not only in terms of their frequency, but also based on their anatomical and isotopic traits, have aroused interest during the last decade, since IADFs have been proved to be useful as proxies to provide high resolution information on eco-physiological plant responses to the environment rather than constraints in tree-ring series construction (De Micco et al., 2016). Nevertheless, much more IADF-focused research is still needed in order to unravel the functional meaning of their formation, since the link with the ecophysiological processes behind their formation as well as to the exact role of external factors priming them have not been completely explained yet (Battipaglia et al., 2016a).

One of the species covering a broad range of Mediterranean area is *Quercus ilex* L. (holm oak). *Q. ilex* forests are distributed throughout the Mediterranean Basin and dominant in a transition zone between temperate forests and shrublands (Terradas, 1999). *Q. ilex* is a monoecious species, blossoming in May-June with new leaf growth (Terradas, 1999); moreover, after dry summers, new leaves in autumn can be developed (Praciak et al., 2013). It is less adapted to extreme drought in comparison with other evergreen Mediterranean tree species such as *Ceratonia siliqua* L., *Olea oleaster* Hoffmanns. & Link and *Quercus suber* L. (Terradas, 1999), but, on the other hand, it is more capable to withstand low temperatures, surviving up to -24 °C in winter for short periods (Knopf, 2002). Moreover, *Q. ilex* is also able to suspend the vegetative growth during drought periods and reactivate it when the water is available again (Del Favero, 2008): its capability of stopping vegetative activities during drought periods creates an irregular pattern of wood formation

and more than one growth-ring can be often produced during a calendar year (the above mentioned IADFs) (Gené et al., 1993; Zhang and Romane, 1991). Therefore, the study of *Q. ilex* tree rings, which received less interest over time compared to the more easily analyzable softwood or temperate species, would be useful to extend the knowledge to a large geographical scale, taking different stand structures and microclimates into account. Hence, IADF frequency and width, as well as IADF anatomical trait analysis in tree rings could be a source of proxy data to reconstruct *Q. ilex* response to intra-annual fluctuations of temperature and precipitation conditions, once the relationship with the triggering factors is found out. *Q. ilex* forests widely occur in pure stands or in mixed forests with Mediterranean pines such as *Pinus halepensis* Mill. or *Pinus pinea* L.. Particularly, mixed forests of *Q. ilex-P. pinea* are extensively found throughout the Mediterranean Basin, being representative of two coexisting functional types: evergreen-sclerophyllous drought-resistant broadleaf and Mediterranean-adapted drought-avoidant conifer. *Q. ilex* is a shade-tolerant tree, becoming dominant in late successional stages; it is deep rooted and has a great capacity to mantain high stomata conductance during drought periods (Barbero, 1992). On the contrary, *P. pinea* has a shallow root system and reduces water use during drought as a drought-avoidant species, being able to recover when water becomes available again (Mayoral et al., 2015; Zalloni et al., 2016). Drought-adaptation ability may be species specific, and even small differences between co-existing species may prove to be decisive for competition and thus for future population development and resilience. The contrasting physiological strategies adopted by *Q. ilex* and *P. pinea* to cope with water stress indicate different sensitivity to temperature and to water availability, thus influencing all the processes regulating their growth. Therefore, wood formation would be ultimately affected, recording climate fluctuations in tree-ring anatomical features such as IADFs: the physiological bases leading to IADFs may be extrapolated through the analysis of the variation of functional wood traits and $\delta^{13}C$ and $\delta^{18}O$ of tree rings at an intra-annual scale (Battipaglia et al., 2010, 2014; De Micco et al., 2007, 2012). This approach, combining quantitative wood anatomy and the analysis of stable isotopes, would be useful to add valuable information on *Q. ilex* and *P.*

pinea mixed forests responses to drought and their ecological role for Mediterranean forest dynamics.

Climate response of tree-species growth could be also influenced by intra- and inter-specific interactions. The different physiological strategies of stress response and resource use among species could improve stand growth performances, as they could also lead to different levels of competition and/or complementarity, which could change along sites and over time with climatic conditions and tree aging (Forrester, 2014). Climate forecasted changes will affect the conservation, productivity and management of forest ecosystems (Ciesla, 1995). Whether pure or mixed stands can be more productive through time depends on abiotic factors such as climatic conditions which, in turn, influence stand development and the availability of light and resources: as stand develops, there are changes in the dominance of species with different growth and physiological strategies in mixed stands which there are not in pure stands (Forrester, 2015). For instance, whether to maintain stand diversity or to push to the natural shifting from mixed forests of *Q. ilex-P. pinea* to *Q. ilex* pure stands is still an open question. The increased intra-annual frequency and duration of drought periods in Mediterranean areas may lead to changes in water use efficiency (WUE) which could facilitate one species in spite of another, i.e. faster growing trees in mixed stands could use more water than slower growing trees (Law et al., 2002; Schume et al., 2004), making them more sensitive to drought than less productive pure stands. Understanding what drives inter- and intra-species interactions under a changing climate is necessary when managing mixed and pure stands, since one of the priorities in forestry is to acquire knowledge on the capability of different forest ecosystems to adapt to short- to long-term climatic variability (Brooker, 2006), especially in climate change hot spots such as the Mediterranean basin. In this context, finding the link between IADF occurrence, which has been linked to intra-annual frequency of dry periods, and facilitation and/or competition effects of different stands, which are linked to changes in climatic conditions, could be useful to add insights in the ecological role of such anatomical traits.

In this framework, the main aims of this thesis are: a) to assess a method allowing to determine *if* and *how* the relationships between IADF frequency and tree-ring age/width vary and to standardize IADF frequency chronologies in order to identify the main climatic factors driving their formation under Mediterranean climate; b) to characterize anatomical traits and carbon and oxygen stable isotope composition in IADFs occurring in tree rings of *Q. ilex* and *P. pinea* trees co-existing at a same site and to investigate whether they can be used as indicators of species-specific responses to intra-annual climate fluctuations; c) to search for the influence of local site conditions on the relationship between IADF occurrence, width and wood anatomical structure and climate in *Q. ilex* tree rings; d) to find a link between IADF occurrence and stand productivity of *Q. ilex* growing in pure and mixed stands with *P. pinea*. To pursue the main aims of the thesis an additional study has been included, in which we built a long *Q. ilex* tree-ring width mean chronology based on old cross section observation, with the aim to help us to date all the younger *Q. ilex* samples of the case studies presented in the thesis. The study cases presented in this thesis refer to a network covering sites in Italy, Portugal and Spain, to stands located within the Somma-Vesuvio volcanic complex, in the Vesuvio National Park, at about 15 km SE of Naples (Southern Italy), and to a stand located in the woodland of the Royal Palace of Portici (Reggia di Portici), Southern Italy.

1.2 Multidisciplinary methodological approach: Dendrochronology, Quantitative Wood Anatomy and Stable Isotope Analysis

Tree rings are one of the main biological proxy climate indicators, allowing high-resolution records of environmental conditions which are important to forecast species and plant performance responses to climate change scenarios (Fritts, 1976; Schweingruber, 1988). Secondary meristems, as well as apical meristems, keep forming new functional tissues during tree's life-span under the influence of physiological internal and environmental external factors. As long-living organisms, trees permanently record in tree rings their responses to the surrounding changing environment, which directly or indirectly affects cambial activity and cell differentiation, having to continuously

adjust their hydraulic system to cope with external stresses during their growth. These responses have often been reconstructed with the use of dendrochronology (Čufar, 2007). Dendrochronology is the science that uses tree rings dated to their exact year of formation to analyze temporal and spatial patterns of processes in the physical and cultural sciences (Henri D. Grissino-Mayer's Ultimate Tree-Ring Web Pages, http://web.utk.edu/grissino/). It is based on the development of time series following the principle of crossdating which allows to identify the exact calendar (or relative) year in which each tree ring was formed, through the matching of ring width (or other ring characteristics) patterns among several tree-ring series. The extrapolation of environmental information from tree-ring series (dendroecology) has its bases in the assumption of the existence of a common signal caused by common biotic or abiotic factors in trees growing under similar environmental conditions, which is shown in their time series as a common variability (Schweingruber, 1996). Tree rings of Mediterranean species have been used for dendroecological purposes less than tree rings of plants growing in temperate environments, because of the difficulty to clearly recognize and date their tree annual rings. In temperate climates, plant cambial activity stops during the cold season, and annual tree rings are easily recognizable on cross sections. Differently, cambial activity may stop not only due to sub-optimal winter temperature, but also because of drought events occurring in summer months in Mediterranean species (the so-called Mediterranean "double stress"), leading to the formation of IADFs in tree rings (Cherubini et al., 2003). Among widespread Mediterranean species, the potential value of *Q. ilex* for dendrochronological and dendroclimatological studies has already been shown by studies in France, Portugal and Spain (Abrantes et al., 2012; Campelo et al., 2007, 2009; Corcuera et al., 2004; Gea-Izquierdo et al., 2009, 2011; Gutiérrez et al., 2011; Patón et al., 2009; Terradas and Savé, 1992; Zhang and Romane, 1991), but tree-ring dating of *Q. ilex* trees is still a complex task which limits the number of studies using this evergreen Mediterranean species rather than others.

Starting with the same principles of dendrochronology, the study of the temporal variability of intra-annual wood-cell anatomical traits, which began already in the second half of the 20th century

(Baas, 1976; Carlquist, 1975, 1980, 1984; Wheeler et al., 2007), has received more attention in the last twenty years thanks to the technological improvement of digital image analysis hardwares and softwares. The processes of cell division and differentiation taking place during tree-ring formation are genetically controlled and depend on the age and size of the tree, but are strongly influenced by environmental conditions (Fonti et al., 2009; Ružička et al., 2015; Scarpella and Meijer, 2004). External stresses, such as drought events, can affect tree physiology which in turn induces wood structure adjustments in order to preserve plant performance and survival (De Micco and Aronne, 2012). The temporal variation of cell-anatomical traits in wood reflects the ability of the species to maintain the balance between mechanical support, water and nutrient transport and storage under environmental stress conditions, with the achievement of a trade-off between efficiency and safety of water transport (Sperry et al., 2008). Therefore, the quantification of anatomical traits of wood cells such as conduit lumen area, wood density, cell wall thickness and vessel frequency (quantitative wood anatomy - QWA) within each ring and quantified through time, provides environmental information with a high-temporal resolution. Mediterranean species, which grow under a climate characterized by the alternation of dry and wet periods, show wood structure adjustments which are mostly related to the achievement of a balance between the need to maintain high conductivity when water is available, and to prevent phenomena of embolism when dry conditions occur (Cherubini et al., 2003; De Micco and Aronne, 2012; Meinzer et al., 2010; Sperry, 2008). This adjustments in wood anatomical traits often lead to the formation of IADFs in tree rings, being the adaptation of cambial activity and xylem differentiation to changes in environmental conditions throughout the growing season considered the main mechanism for IADF formation (De Micco et al., 2016). QWA methodological approach based on tree-ring time series analyzed with dendrochronology techniques can contribute to shed light on the relationships between functional traits and the adaptive capability of the species, thus to the estimation of the impact of forecasted climate changes on future species distribution dynamics. The analysis of anatomical traits of *Q. ilex* tree rings in some studies showed the potentiality of its wood features

for climate reconstruction in the Mediterranean region (Abrantes et al., 2013; Campelo et al., 2010; Corcuera et al., 2004; Villar-Salvador et al., 1997). To the best of our knowledge, IADFs in tree rings of *Q. ilex* have not been characterized yet in terms of anatomical traits. Moreover, it is still not clear how the occurrence of peculiar wood anatomical traits in tree rings, such as the IADFs, is linked to species drought adaptation ability and stand performance. However, IADFs could be used as indicators of specific physiological and ecological behavior of trees once the link between their formation and appearance and climate is found out.

The link between xylem hydraulic properties and the related physiological mechanisms can be investigated through wood stable isotope analysis. The quantification of the $^{13}C/^{12}C$ and the $^{18}O/^{16}O$ composition of tree rings helps to reconstruct past atmospheric CO_2 and soil water assimilation of the plant, whose measure is related to tree response to the environment. The values of the ratios reflect the amount in which the heavier isotope is discriminated compared to the lighter one during the physical and chemical processes involved in the synthesis of plant organic matter (Farquhar et al., 1989). The $\delta^{13}C$ value can vary due to both photosynthetic rate and stomatal conductance, since both of them affect the ratio between CO_2 partial pressure in leaf intercellular space and in the atmosphere. Wood $\delta^{18}O$ value, instead, can provide information about stomatal conductance, since it is influenced by soil water, which is in turn related to precipitation isotope composition, residence time in the soil and evaporation effects, and it is also inversely related to the ratio between atmospheric and leaf intercellular water vapour pressure (Farquhar et al., 1982; McCarroll and Loader, 2004). Therefore, coupling the measurement of wood carbon and oxygen stable isotope composition helps understanding whether CO_2 assimilation is more strongly affected by biochemical or stomatal constraints, as explained by the conceptual model showed by Scheidegger et al. (2000). In the context of this thesis, the physiological bases leading to IADFs could be extrapolated by the analysis of functional wood traits and $\delta^{13}C$ and $\delta^{18}O$ of tree rings at an intra-annual scale (Battipaglia et al., 2010, 2014; De Micco et al., 2007, 2012). Intra-annual QWA and stable isotope analysis were successfully combined together to study IADFs of *P. pinaster* Aiton.

(De Micco et al., 2007) and of *Arbutus unedo* L. (Battipaglia et al., 2010); to the best of our knowledge, there is no such a characterization of IADFs in tree rings of *Q. ilex* neither of *P. pinea.* Variations in stable isotope composition of co-occurring plant species may reflect functional diversity of water use strategies (Ferrio et al., 2003), thus can help to shed light on the link between the formation of peculiar wood anatomical traits such as IADFs and their functional and ecological role. $\delta^{13}C$ can be used to calculate an intrinsic index of WUE (WUE_i) (Altieri et al., 2015; Battipaglia et al., 2016b; Dawson and Ehleringer, 1993; Moreno-Gutiérrez et al., 2012), which is the key for the survival of Mediterranean species under drought conditions (Farquar et al., 1989): the increase in WUE_i can be considered as an adaptive trait of Mediterranean species (Medrano et al., 2009). Inter- and intra-species interactions influence WUE_i, and vary with inter- and intra-annual climatic variability, stand density, tree size (Forrester, 2015). The analysis of the WUE_i and wood $\delta^{18}O$ together with their relative complementarity indexes, could reveal differences between photosynthetic rate and stomatal conductance balance of *Q. ilex* growing in pure or in mixed stands, which could be linked to the formation of more or less IADFs in tree rings.

1.3 Intra-Annual Density Fluctuations: What do they are?

IADFs are abrupt changes in density within a tree ring, and are frequently found in Mediterranean species as a response to seasonal climate alternation between dry and wet periods (Cherubini et al., 2003, De Micco et al., 2016). Contrarily to environments where trees often form annual rings, since cambial activity lasts a single period and stops once a year due to a limiting factor such as extreme low or high temperatures, IADFs are formed when cambial activity stops more than once a year due to peculiar seasonal temperature and/or precipitation fluctuations, leading to complex tree rings with several successive layers of earlywood and latewood (Bräuning 1999; Cherubini et al. 2003; Rigling et al. 2001; Schulman 1938; Tingley 1937). Observed under the microscope at low magnification or with naked eye, they appear as wood growth bands of latewood-like cells within the earlywood or of earlywood-like cells within the latewood (Fritts, 1976). Different types of

IADFs can be found in different species as well as in the same tree in different years or in the same year in different trees. IADF identification is easier in softwoods, where the distinction between earlywood and latewood is clearer, than in hardwoods, especially diffuse-porous woods, where density fluctuations within the ring may be related to the variation of several anatomical traits such as lumen area and cell-wall density of vessels as well as of fibres or parenchyma. Since the distinction between IADFs and true ring boundaries is not always easy in hardwoods, most of the studies on IADFs have been focused on coniferous species until recent days. However, thanks to the progress in identifying and characterising the IADFs, which took place during the last decade, what were previously considered as constraints in the application of dendrochronology in certain species such as *Q. ilex*, are now considered as traits which may instead help to crossdate tree-ring series and to study climate influence on tree growth at an intra-annual level (De Micco et al., 2016). In Mediterranean species, IADF frequency, although related to tree age, size or tree-ring width as well as species genetic and site conditions, depends on drought: it is the result of wood functional trait adjustments induced by Mediterranean "double stress", in order to preserve hydraulic conductivity (Cherubini et al., 2003, De Micco et al., 2016). The ability to adapt to the highly seasonal Mediterranean conditions may depend on the ability of the species to adjust cambial activity in order to cope with the prevailing environmental condition forming IADFs, since trees that are not capable to quickly adjust their wood traits to respond to climate are more vulnerable to drought (Martinez-Meier et al., 2008). Nevertheless, the question whether they result from an hydraulic structure adjustment of trees to avoid stressful conditions or to take advantage of favorable ones is still open (Battipaglia et al., 2016a). The most of the IADF-focused studies investigate the climate signal of IADF frequency, while less is investigated with the analysis of the intra-ring variability of anatomical traits, also combined with stable isotope analysis of IADFs. Given the dependence of IADF frequency on cambial age, a detrending method was performed (Zalloni et al., 2016, Chapter 1), which allowed also to overcome limitations due to local replication. IADFs can be used as indicators of past climatic conditions with a higher resolution than annual tree rings, once the

relationships between IADF occurrence and type and climatic conditions for the different species are found out.

1.4 Main aim

The general aim of this dissertation was to find a link between structure and function of Intra-Annual Density Fluctuations in Mediterranean species tree rings, focusing on *Quercus ilex* L. which may help in the study of the influence of climatic factors on tree growth. This study would provide information on the ecological behavior and vulnerability of the species, leading to an improvement of the implementation of forest management options.

1.5 Study cases and related specific objectives

Chapter 1

This chapter reports the investigation of the effect of age, tree-ring width and climate on IADF formation and frequency at a regional scale across the Mediterranean Basin in *P. pinea*, *Pinus pinaster* Ait. and *Pinus halepensis* Mill. trees growing in Italy, Portugal and Spain, in order to standardize IADF frequency chronologies and to identify the main climatic factors driving their formation under Mediterranean climate.

Chapter 2

This chapter reports the characterization of the anatomical traits and the carbon and oxygen stable isotope composition of IADFs occurring in tree rings of *Q. ilex* and *P. pinea* trees co-existing at a same site in Southern Italy, and the relationship between their occurrence and seasonal mean temperature and total precipitation, in order to investigate whether they can be used as indicators of species-specific responses to intra-annual climate fluctuations.

Chapter 3

This chapter reports the investigation of growth response and of the relationship between IADF occurrence, IADF width and IADF wood anatomical traits and temperature and precipitation of *Q.*

ilex populations living on two opposite slopes in the National Park of the Vesuvius, Southern Italy, in order to search for the influence of local site conditions on the climate signal of IADF occurrence and IADF anatomical structure.

Chapter 4

This chapter reports the comparison of the temporal variation of cumulative basal area, WUE_i assessed through $\delta^{13}C$ in tree rings and $\delta^{18}O$, and of IADF frequency within tree rings of *Q. ilex* growing in a pure and in a mixed stand with *P. pinea* in two study areas differing from stand characteristics in Southern Italy, in order to assess whether pure or mixed *Q. ilex* stands can be more productive through time depending on climatic conditions and stand structure, and to find a link between IADFs, stand productivity and stand structure.

Chapter 5

This chapter reports the development of a long *Q. ilex* tree-ring width mean chronology based on old cross section observation, with the aim to help us to date all the younger *Q. ilex* samples of the previous case studies.

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 02 May 2016
doi: 10.3389/fpls.2016.00579

2.

Climatic Signals from Intra-annual Density Fluctuation Frequency in Mediterranean Pines at a Regional Scale

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *22 December 2015*
Accepted: *14 April 2016*
Published: *02 May 2016*

Citation:
Zalloni E, de Luis M, Campelo F, Novak K, De Micco V, Di Filippo A, Vieira J, Nabais C, Rozas V and Battipaglia G (2016) Climatic Signals from Intra-annual Density Fluctuation Frequency in Mediterranean Pines at a Regional Scale. Front. Plant Sci. 7:579. doi: 10.3389/fpls.2016.00579

Tree rings provide information about the climatic conditions during the growing season by recording them in different anatomical features, such as intra-annual density fluctuations (IADFs). IADFs are intra-annual changes of wood density appearing as latewood-like cells within earlywood, or earlywood-like cells within latewood. The occurrence of IADFs is dependent on the age and size of the tree, and it is triggered by climatic drivers. The variations of IADF frequency of different species and their dependence on climate across a wide geographical range have still to be explored. The objective of this study is to investigate the effect of age, tree-ring width and climate on IADF formation and frequency at a regional scale across the Mediterranean Basin in *Pinus halepensis* Mill., *Pinus pinaster* Ait., and *Pinus pinea* L. The analyzed tree-ring network was composed of *P. pinea* trees growing at 10 sites (2 in Italy, 4 in Spain, and 4 in Portugal), *P. pinaster* from 19 sites (2 in Italy, 13 in Spain, and 4 in Portugal), and *P. halepensis* from 38 sites in Spain. The correlations between IADF frequency and monthly minimum, mean and maximum temperatures, as well as between IADF frequency and total precipitation, were analyzed. A significant negative relationship between IADF frequency and tree-ring age was found for the three Mediterranean pines. Moreover, IADFs were more frequent in wider rings than in narrower ones, although the widest rings showed a reduced IADF frequency. Wet conditions during late summer/early autumn triggered the formation of IADFs in the three species. Our results suggest the existence of a common climatic driver for the formation of IADFs in Mediterranean pines, highlighting the potential use of IADF frequency as a proxy for climate reconstructions with geographical resolution.

Keywords: IADF, tree rings, climate, *Pinus halepensis*, *Pinus pinea*, *Pinus pinaster*

INTRODUCTION

Tree-ring width is a powerful proxy of past environmental conditions able to record fluctuations of biotic and abiotic factors during the tree's lifetime (Fritts, 2001). Tree rings reveal physiological response to environmental fluctuations because the latter affect xylogenesis which in turn can lead to peculiar anatomical features, such as intra-annual density fluctuations (IADFs). IADFs are defined as a layer of cells within a tree ring identified by different shape, size, and wall thickness (Kaennel and Schweingruber, 1995), and characterized by the occurrence of latewood-like cells within earlywood or earlywood-like cells within latewood (Fritts, 2001). They can occur in several species in different environments and are often irregularly found in time and space (Cherubini et al., 2003; De Micco et al., 2016). IADFs constitute a useful tool to reconstruct intra-annual changes in climatic factors, providing detailed information at the seasonal level (Rigling et al., 2001, 2002; Copenheaver et al., 2006; Campelo et al., 2007, 2013; de Luis et al., 2007, 2011a; Bogino and Bravo, 2009; Hoffer and Tardif, 2009; Battipaglia et al., 2010; Edmonson, 2010; Vieira et al., 2010; Rozas et al., 2011; Olivar et al., 2012; Novak et al., 2013a,b; Nabais et al., 2014; Olano et al., 2015). IADF formation can be considered as a strategy of trees to adjust wood anatomical traits to short-term variations in environmental conditions maintaining the balance between hydraulic efficiency and safety against embolism during wet and dry periods, respectively (Campelo et al., 2007; De Micco et al., 2007; De Micco and Aronne, 2009; Wilkinson et al., 2015). Numerous studies reported high IADF frequency in species growing in the Mediterranean area, which is considered one of the most vulnerable regions to climate changes. According to the Intergovernmental Panel on Climate Change [IPCC] (2014), higher irregularities in the intra-annual precipitation patterns and increasing temperature are expected in the Mediterranean Basin in the next decades (Giorgi and Lionello, 2008). The expected climate changes will likely have an impact on tree growth and thus IADF frequency.

Most dendrochronological studies on IADF occurrence in the Mediterranean area have been conducted on *Pinus* species, since Mediterranean pines are quite sensitive to climate fluctuations and are prone to form IADFs (Campelo et al., 2007, 2013, 2015; de Luis et al., 2007, 2011a; Carvalho et al., 2015; De Micco et al., 2007; Vieira et al., 2009, 2010, 2015; Rozas et al., 2011; Olivar et al., 2012; Novak et al., 2013a,b; Nabais et al., 2014; Carvalho et al., 2015). Despite the variety of climatic conditions throughout the Mediterranean Basin, *Pinus* is a widespread genus (Barbéro et al., 1998), allowing to compare the climate response of different species at a regional scale.

IADF formation is reported to depend on tree age, sex, size, and/or width of the formed tree-ring (Rigling et al., 2001; Wimmer, 2002; Campelo et al., 2007, 2013, 2015; Bogino and Bravo, 2009; de Luis et al., 2009; Vieira et al., 2009; Olivar et al., 2012; Nabais et al., 2014; Olano et al., 2015). As a consequence, a wide variability in the occurrence of IADFs across species distribution is commonly described (Rigling et al., 2002; Edmonson, 2010; Novak et al., 2013b; Nabais et al., 2014). A higher frequency of IADFs has been found in young trees of *Pinus pinaster* growing under Mediterranean climate compared to older ones (Bogino and Bravo, 2009; Vieira et al., 2009). A similar age-relation has been observed in *Pinus halepensis* stands throughout its natural distribution area in the Iberian Peninsula (Olivar et al., 2012; Novak et al., 2013b). In the Iberian Peninsula, an age and size dependency of IADF frequency in *P. halepensis* and *P. pinaster* trees has been reported: the maximum frequency of IADFs was observed during the juvenile stages (about 27 years-old trees), and more IADFs were found in wider than narrower tree rings (Novak et al., 2013b; Campelo et al., 2015). In *P. pinaster* from east-central Spain, the presence of IADFs has been negatively correlated with radial growth rates (Bogino and Bravo, 2009), while no significant relationships of IADF frequency with age and tree-ring width have been found in young trees (<55 years) from the wetter north-western Spain (Rozas et al., 2011). Aside from Mediterranean pines, significant relationships between IADF frequency and either tree-ring age (negative) or tree-ring width (positive) have been found in *Pinus sylvestris* trees growing in dry sites in the central Alps (Rigling et al., 2001, 2002). Analyzing tree rings of *Pinus banksiana* and *Picea mariana* from eastern Manitoba, Hoffer and Tardif (2009) showed a higher frequency of IADFs in juvenile rings than in older ones, but no significant relation between IADF occurrence and tree-ring width was found.

A few studies have been performed on the geographical variation of IADF occurrence. A significant variability in the frequency of IADFs across the range of *P. halepensis* was found in Spain (Novak et al., 2013b), with a higher frequency of IADFs in coastal sites than inland or mountain sites. Moreover, Rozas et al. (2011) found that IADF frequency of *P. pinaster* under Atlantic climate depends strongly on elevation, with more abundant IADFs at low elevations. Rigling et al. (2002) showed a higher mean IADF frequency in *P. sylvestris* growing at a drier than moderate wet sites in Switzerland. A recent study on *P. pinaster* and *Pinus pinea* comparing a Mediterranean and a temperate site in Portugal highlighted that local adaptation and site-specific climatic conditions can play an important role in the formation of IADFs regardless of the species (Nabais et al., 2014).

The literature survey reveals that available data about the relations between IADFs and climate were based on single species or when more than one species was used they were restricted to a single or a few sites. Indeed, studies based on a network of IADFs covering a broad geographical area would likely help to gain information on the ability of tree species to adjust their hydraulic architecture and physiology in response to intra-annual environmental changes on a larger geographical scale.

In the present study, we used a network of IADF frequency covering a broad geographical area with the aim to analyze whether the occurrence of IADFs in Mediterranean pine species is triggered by common regional climatic drivers. In order to reach this aim, we investigated the relationships between IADF frequency and tree-ring age, tree-ring width and climate in three widespread Mediterranean pine species, namely *P. halepensis*, *P. pinaster* and *P. pinea*, growing along their distribution ranges.

Our specific goals were: (1) to characterize the regional patterns of IADF frequency in *P. halepensis*, *P. pinaster,* and *P. pinea* growing along their distribution range, (2) to determine *if* and *how* the relationships between IADF frequency and tree-ring age/width vary between the three species, and (3) to identify the large-scale climatic factors driving the formation of IADFs under Mediterranean climate, by analyzing the relationships between IADF frequency and monthly maximum temperature (T_{max}), mean (T_{mean}) and minimum temperature (T_{min}), as well as total precipitation.

MATERIALS AND METHODS

The Dataset: Species and Sites

The database consists of 55 previously published and 13 newly processed chronologies of tree-ring width and series of IADF frequency from: (a) *P. pinea* trees growing at 10 sites (i.e., 2 in Italy, 4 in Spain, and 4 in Portugal), (b) *P. pinaster* trees from 19 sites (i.e., 2 in Italy, 13 in Spain, and 4 in Portugal), and (c) *P. halepensis* trees from 38 sites in Spain. Details of each site are reported in the supporting material (Supplementary Table S1).

Climatic time series of monthly temperature and total precipitation for the period 1901–2013, for all the sites, were derived from the Climatic Research Unit (CRU) TS v. 3.22 dataset with 0.5° grid resolution (Harris et al., 2014). CRU gridded data were chosen for comparative purposes because of its complete coverage of all studied sites and high correlations with the local weather stations. Mean monthly temperature and total precipitation as average of all the sites for each species are shown in climate diagrams in **Figure 1**. The overall climate regime is Mediterranean-like, with the occurrence of mild winter and spring, and a period of summer drought followed by an increase in precipitation concomitant to a decrease of temperature during autumn. The mean monthly temperature for all the study sites ranged from 7°C in January to about 24°C in August. The sites with the highest amount of precipitation were those where *P. pinaster* is dominant (**Figure 1B**): at these sites, the highest monthly values of precipitation during the entire year were recorded with a maximum in December (132.3 mm) and a minimum in July (18.8 mm). The lowest values of mean precipitation throughout the year were recorded for *P. halepensis* sites with a maximum of 55.6 mm in October and a minimum of 13.3 mm in July (**Figure 1A**). Finally, *P. pinea* trees grow in sites with the lowest amount of summer precipitation (**Figure 1C**) with July as the driest month (10.8 mm), and a maximum of rainfall in December (73.2 mm).

Identification of IADFs

The database includes three species (*P. halepensis*, *P. pinaster,* and *P. pinea*) from a wide variety of sites. Despite the great potential of IADFs as proxies, the methods for their objective classification in different types (e.g., based on their relative position within the tree ring) have not been standardized yet. At present, to study IADFs, tree-ring series are mainly analyzed visually. Although, tree-ring width measuring and IADF identification were performed by different operators, all followed a common protocol and all were trained to adopt the same criteria for IADF identification. This allowed unambiguous identification of the presence/absence of IADFs, but the classification of different types of IADFs still suffered from subjectivity. Consequently, to build series of IADF occurrence, we adopted a conservative criterion using only the presence/absence of IADFs in tree rings visually identified on dated cores with the help of a stereomicroscope.

Relationships between IADF Frequency and Tree-Ring Age and Width

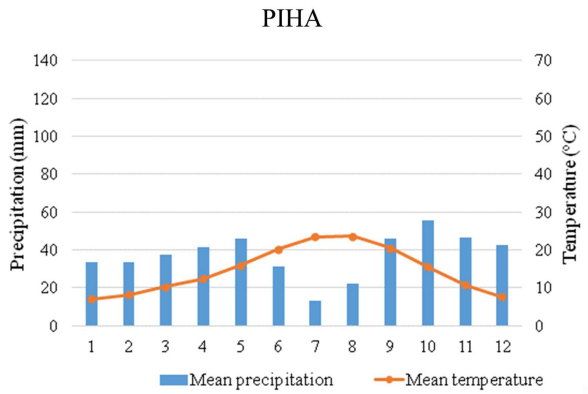
The age of individual tree rings (here, defined as "tree-ring age") was indicated in ascending order starting from the most juvenile ring to the oldest one within each core. To study the influence of tree-ring age on the likelihood of IADF formation, a logistic binomial model was applied using tree-ring age as the independent variable and the presence (1) or absence (0) of IADFs in the corresponding tree ring as the dependent variable. The analysis was conducted independently for each species and was limited to tree-ring ages with at least 20 tree rings. Data from a total of 84,794 tree rings ranging from tree-ring age 1 to 169 were included in the analysis for *P. halepensis*. The total number of tree rings analyzed for *P. pinaster* and *P. pinea* was 30,792 and 16,028, while the range of tree-ring age was from 1 to 125 years and from 1 to 108, respectively. Predicted values of IADF frequency obtained for each age class were used as reference series for detrending purposes.

The influence of tree-ring width on the likelihood of IADF formation was analyzed with a similar procedure and the same dataset by using a new set of logistic binomial models with the width of each individual tree ring as the independent variable. Predicted and detrended IADF values for each individual tree-ring width were calculated with the same method previously described for tree-ring age.

IADFs Frequencies and Geographical Pattern

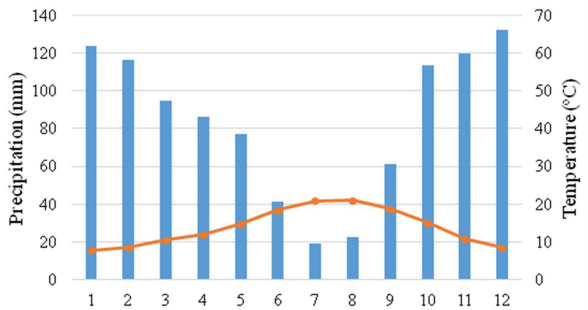
The geographical pattern of IADF frequency was analyzed using age-detrended IADF values obtained from the logistic binomial models including tree-ring data from all three species. To obtain detrended IADF values, the ratio between observed (0 or 1) and predicted IADF frequencies ([0,1]) was calculated for each individual IADF observation. Then, to obtain a robust estimation of the frequency of IADFs, independent from the age structures of the studied populations, the average of all individually obtained ratios (thereafter referred as IADF_r) were calculated for each study site. For each species, IADF_r equal to 1 represents years in which the frequency of IADFs is equal to the expected species-specific average. IADF_r of 2 and of 0.5 indicates that IADF frequency was twice and half the expected average, respectively. IADF_r were then rescaled to allow intra- and inter-species comparison. To do that, IADF_r obtained for each population was multiplied by the average IADF as predicted from age 1 to age 100 of the specific logistic model. The obtained rescaled frequency (IADF_f) represents the estimated frequency of IADFs

A



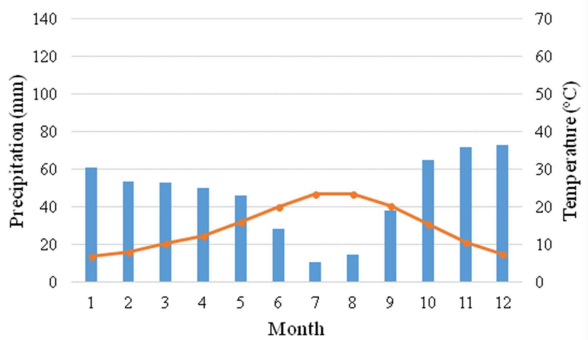
PIHA	Mean precipitation (mm)	Mean temperature (°C)
Annual	465 ± 94	14 ± 2
Winter	114 ± 31	7 ± 2
Spring	130 ± 29	13 ± 2
Summer	71 ± 34	22 ± 2
Autumn	150 ± 27	15 ± 2

B



PIPI	Mean precipitation (mm)	Mean temperature (°C)
Annual	1111 ± 392	14 ± 1
Winter	401 ± 156	8 ± 2
Spring	273 ± 89	12 ± 2
Summer	101 ± 39	20 ± 2
Autumn	336 ± 118	15 ± 2

C



PIPN	Mean precipitation (mm)	Mean temperature (°C)
Annual	569 ± 144	16 ± 2
Winter	199 ± 75	9 ± 3
Spring	148 ± 30	14 ± 2
Summer	47 ± 32	23 ± 1
Autumn	176 ± 51	17 ± 2

FIGURE 1 | Climate diagrams and seasonal and annual values of mean precipitation and mean temperature of the sites of the network grouped by species, for the period 1901–2013 [(A) *Pinus halepensis* (PIHA), (B) *Pinus pinaster* (PIPI), and (C) *Pinus pinea* (PIPN)]. Data from the CRU TS v. 3.22 dataset with 0.5° grid resolution (Harris et al., 2014).

for each population which is independent from the population age structure and comparable between sites and species.

Replication Depth and a New Approach to Study Climatic Signal in IADFs

The principle of replication represents one of the keys of dendrochronological research highlighting the need to use more than one stem radius per tree and more than one tree per site to obtain reliable tree-ring chronologies. Different statistics based on mean inter-correlation among tree-ring series, as the expressed population signal (EPS), which determines how well a chronology established on a finite number of trees approximates the theoretical population chronology (Wigley et al., 1984; Briffa and Jones, 1990), are often used to identify well-replicated periods for different types of dendrochronological series (e.g., width, density, or chemical composition). Sampling strategies in dendrochronology are often designed to ensure such replication requirements.

However, the presence of other anatomical features like IADFs cannot be measured but just characterized as a binary variable of 0 and 1 (dummy variable), based on its absence or presence in a specific tree ring. In these cases, criteria to define the appropriate number of samples to obtain an accurate representativeness of IADF frequency cannot be based on the same approach used for tree-ring chronologies, due to the binary nature of the data. To determine the appropriate sample size needed to estimate the proportion of a population that possesses a particular property (i.e., IADF occurrence), a specific calculation needed to be computed (Eq. 1).

This equation allowed calculating the required sample size in order to estimate a proportion (prevalence) with a specified level of confidence and precision. For example, the number of required samples to estimate IADF frequency for a given site and a specific year, with a 95% of confidential level ($z = 1.96$) and a precision of 10% ($e = 0.1$), is 97 (Eq. 1).

$$n = (z^2 * p(1-p))/e^2 = [1.96]^2 * 0.5(1-0.5)/[0.1]^2 = 97 \quad (1)$$

Indeed, this number is substantially higher than the number of samples that are commonly collected in dendrochronological research (based usually on 15 trees and 2 samples per tree). Thus, replication depth issue represents an important challenge aimed to obtain reliable estimations of the frequency of anatomical variables such as IADFs, especially when the aim is to identify the main climate factors promoting their formation. A well-defined sampling strategy could be the perfect solution to reach this purpose.

Nevertheless, to deal with this challenge we adopted an alternative analytical approach which allowed us to use datasets already available (previously collected for other dendrochronological purposes), but solving the problem associated to the high replication depth required.

Our approach was based on a global analysis by combining information from all the study sites and years. To study the influence of annual precipitation on IADF formation in a given species, all available individual tree rings were grouped in 100 classes according to the percentile positions of the local annual precipitation of the year of their formation. Tree rings were grouped in classes ranging from the ones formed under drier to those formed under wetter conditions. Then, mean annual precipitation and mean standardized IADF frequencies were calculated for each class. The statistical normality of the obtained IADF series was verified using the Kolmogorov–Smirnov's test, then Pearson's correlation coefficient was computed to study the association between the two series. By using such procedure, IADF frequencies were not calculated independently for any specific calendar year but estimated for different ranges of annual precipitation conditions. The estimation of frequency associated to each precipitation class was based on at least 158 samples (as for *P. pinea*), in agreement with replication requirements, since dataset including IADF quantification and climate data (1901–2013) included 79901, 30736, and 15889 tree rings for *P. halepensis*, *P. pinaster,* and *P. pinea,* respectively.

Furthermore, since IADF frequencies were not calculated on time series of tree rings in chronological order, but by grouping rings in classes according to the climate conditions occurring during their formation, autocorrelation did not affect the significance level of the results.

The same procedure as explained for annual precipitation was also applied to mean annual temperature (T_{mean}), minimum (T_{min}) and maximum temperature (T_{max}) and total precipitation at monthly and seasonal scales from September of previous year to December of the current year. The correlations with temperature and precipitation of previous autumn months were performed to investigate the effect of growth conditions of the previous year on IADF frequency. The months of the whole calendar year were chosen for correlations between IADF frequency and current growth conditions, since cambial activity under Mediterranean climate was found to be active up to December (de Luis et al., 2009, 2011a,b).

RESULTS

Descriptive statistics and a summary for the measured variables from the three species are shown in **Table 1**. A total of 139,342 rings were analyzed for the three species considered together, of which 24,143 showed IADFs. Mean age varied among species and ranged between 38 and 48 years. Mean tree-ring width ranged between 1.77 mm for *P. halepensis* and 2.76 mm for *P. pinaster*.

IADF Frequency and Tree-Ring Age

An age-dependent trend was found in the distribution of IADF frequency for all the analyzed species (**Figures 2A–C**). Higher frequency of IADFs was found in juvenile than older rings in the three Mediterranean pines, with the peak shifting to different ring ages depending on the species. The logistic binomial regression between tree-ring age and the IADF frequency showed an asymmetric bell-shaped distribution with a maximum of 12% at the age of 26 years in *P. halepensis*, of 45.9% at the age of 19 years in *P. pinaster* and of 26.9% at the age of 38 years in *P. pinea*. Sample depth per each species is shown in **Figure 2**.

IADF Frequency and Tree-Ring Width

The analysis of IADF frequency related to tree-ring widths showed a similar tendency of the three pine species with the occurrence of more IADFs in wide rings than in narrow

TABLE 1 | Descriptive statistics and measured variables for the three species (PIHA, *Pinus halepensis*; PIPI, *Pinus pinaster*; PIPN, *Pinus pinea*).

	N of rings analyzed	Mean age (years)	Mean tree-ring width (mm)	N of rings with IADFs (raw frequency of IADFs)
PIHA	88375	48	1.77	7333 (0.08)
PIPI	33851	38	2.76	13161 (0.39)
PIPN	17116	45	2.36	3649 (0.21)

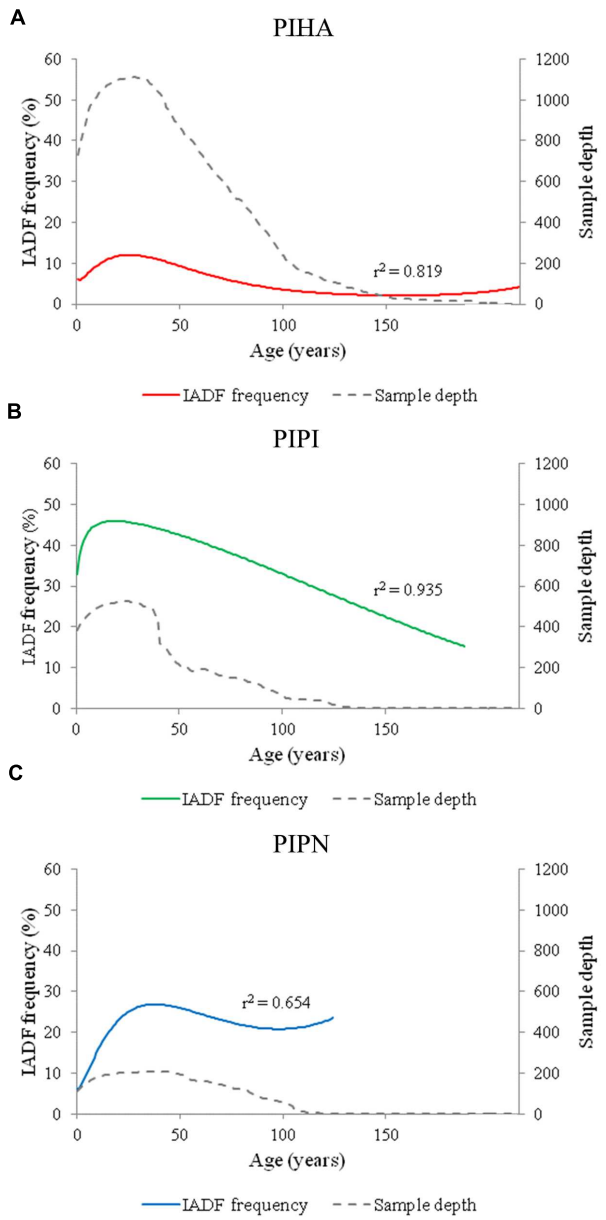


FIGURE 2 | Logistic binomial regressions between IADF frequency and tree-ring age for the three species (PIHA, *Pinus halepensis*; PIPI, *Pinus pinaster*; PIPN, *Pinus pinea*). Population structure and IADF frequency for **(A)** *Pinus halepensis* (PIHA), **(B)** *Pinus pinaster* (PIPI), and **(C)** *Pinus pinea* (PIPN). Age refers to tree-rings aligned from the most juvenile to the oldest ones.

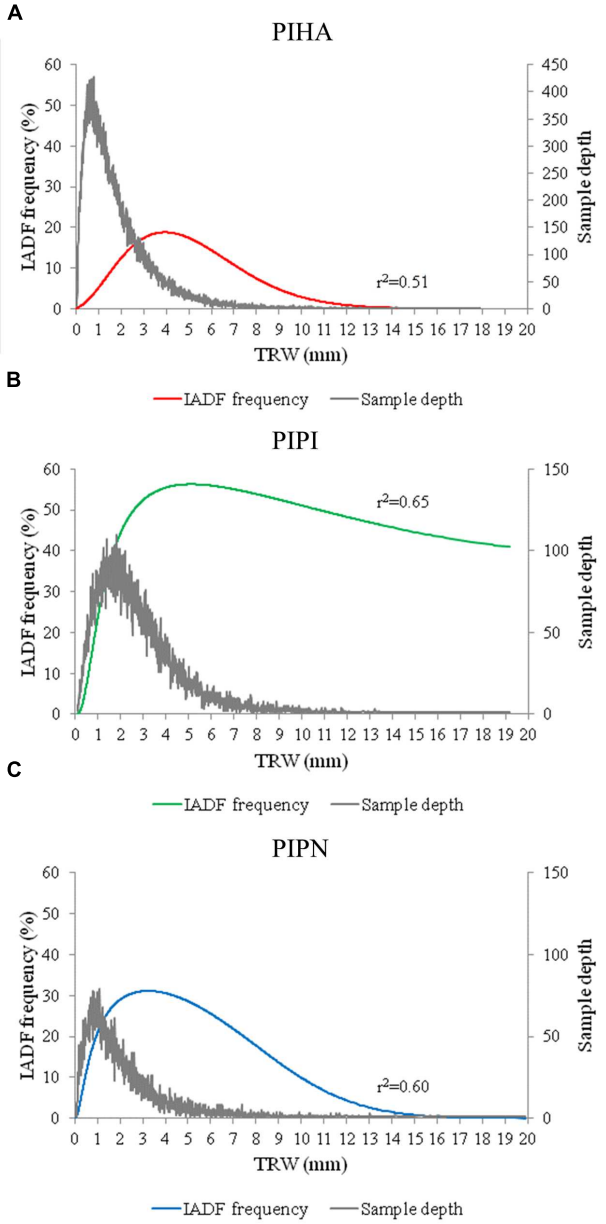


FIGURE 3 | Logistic binomial regressions between IADF frequency and tree-ring width for the three species (PIHA, *Pinus halepensis*; PIPI, *Pinus pinaster*; PIPN, *Pinus pinea*). IADF frequency and number of samples related to tree ring width for **(A)** *Pinus halepensis* (PIHA), **(B)** *Pinus pinaster* (PIPI), and **(C)** *Pinus pinea* (PIPN). TRW refers to tree-rings aligned from the narrowest to the widest ones.

or very large rings, especially in *P. halepensis* and *P. pinea*. Conversely, in *P. pinaster*, despite the decline showed in the largest tree rings, the frequency of IADFs was maintained above 40% in rings wider than 1 cm (**Figures 3A–C**). The highest IADF frequencies were observed for tree rings representing the percentiles 0.91, 0.89, and 0.80 of their ring widths for *P. halepensis*, *P. pinaster,* and *P. pinea*, respectively. The distributions of IADF frequency in relation to tree-ring width are bell shaped for all the three species. The comparison of IADF frequency and tree-ring width with sample depth showed that the highest values of IADF frequency are in the same range of ring widths (3–5 mm) for the three species regardless of the different growth rates, and it shows the increase in tree-ring width moving from the narrowest rings of *P. halepensis* to the widest ones of *P. pinaster* (**Figures 3A–C**).

Geographical Pattern of IADF Frequency

The spatial distribution of mean IADF frequencies standardized by age showed a geographical pattern of fluctuations in the entire network (**Figure 4**). Data of raw and detrended frequency for each site are shown in the supporting material (Supplementary Table S2). *P. halepensis* in Spain was the species with the narrowest range of IADF frequencies, with a minimum of 0.3% and a maximum of 34.9% (**Figure 4**). *P. pinaster* showed the widest range of frequencies of IADFs ranging between 6.8 and 93.2%, with the highest values of frequency in north-west Spain (**Figure 4**). Finally, *P. pinea* IADF frequencies ranged between 2.2 and 53.6%, with the lowest values in Spain and maximum values in Portugal and in Italy (**Figure 4**).

IADF Frequency and Climate

In all the three species, autumn precipitation of the current growth year seemed to be the main climatic condition triggering IADF formation (**Figures 5A–C**). Correlation coefficients between precipitation in autumn and IADF frequency were 0.4 in *P. halepensis*, 0.8 in *P. pinaster,* and 0.7 in *P. pinea* ($p < 0.05$). Significant negative correlations with precipitation were found in June in *P. halepensis* ($r = -0.3$) and in July in *P. pinea* ($r = -0.3$), while *P. pinaster* IADF frequency was positively correlated with precipitation during the whole year ($p < 0.05$). IADF frequency was positively correlated with temperature throughout the year in *P. halepensis*, with values of 0.5–0.7 for T_{min}, 0.5–0.7 for T_{max}, and 0.5–0.7 for mean temperature. IADF frequency was also positively correlated with temperature throughout the year in *P. pinea*, with values ranging of 0.3–0.7 for T_{min}, 0.1–0.7 for T_{max}, and 0.3–0.7 for mean temperature. By contrast, a highly significant negative correlation with summer temperatures (from June to September) was observed in *P. pinaster* ($r = -0.5$ with T_{min}, $r = -0.6$ with T_{max}, and $r = -0.6$ with mean temperature), where the most negative correlations were found in July ($r = -0.5$ with T_{min}, $r = -0.6$ with T_{max}, and $r = -0.6$ with mean temperature; **Figure 5C**). Maximum autumn temperature also showed a negative correlation with IADF frequency in *P. pinaster* ($r = -0.3$), with a high negative correlation in September ($r = -0.2$ with T_{min}, $r = -0.6$ with T_{max}, and $r = -0.4$ with mean temperature).

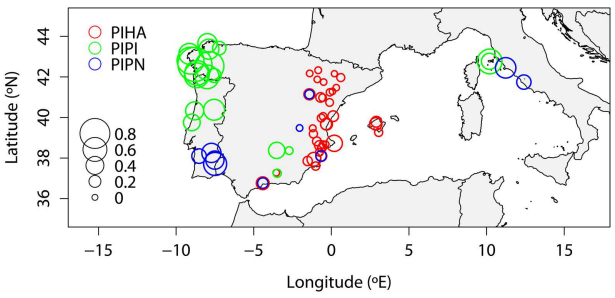


FIGURE 4 | Map of the mean IADF frequencies (IADF_f) of the sites of the network detrended by age (x axis: longitude –W, +E). The amplitude of the circles is directly proportional to the frequency: wider circles are related to higher IADF frequency compared to smaller ones (PIHA, *Pinus halepensis*; PIPI, *Pinus pinaster*; PIPN, *Pinus pinea*).

DISCUSSION

IADF Frequency – Tree-Ring Age Relationship

This study based on a large number of samples throughout the western Mediterranean Basin confirmed the presence of a strong relationship between IADF frequency and tree-ring age in all the analyzed pine species. An age trend toward a higher formation of IADFs in juvenile rings than in older ones was in agreement with previous studies showing that both tree-ring width and IADF frequency are age-dependent (Rigling et al., 2001, 2002; Bogino and Bravo, 2009; Hoffer and Tardif, 2009; Vieira et al., 2009; Olivar et al., 2012; Novak et al., 2013b; Campelo et al., 2015). A different timing and duration of xylem formation may explain the age-dependent IADF frequency. Indeed, the high frequency of IADFs in juvenile tree rings could be due to an earlier reactivation of the cambium and the consequent longer growing season, together with a fast physiological and morphological response to changing factors within the growing season (Villalba and Veblen, 1994; Vieira et al., 2009). On the other hand, high IADF frequency in young individuals could also be attributed to a higher sensitivity to environmental fluctuations: the shallower root systems of younger trees would favor IADF formation in response to changing water availability (Ehleringer and Dawson, 1992; Battipaglia et al., 2014). The relationship between a higher IADF frequency in tree rings and a shallower root system was also found by Pacheco et al. (2016), suggesting a higher sensitivity of the shallower rooted Spanish juniper to summer and autumn rains compared to the deeper rooted Aleppo pine in northeastern Spain. The strong relationship between IADF frequency and age highlights the necessity to overcome age trends in order to have an independent reconstruction of climate from IADFs (Novak et al., 2013b). In this paper, we show the importance of using a standardization method to obtain IADF series without the effect of the population structure and comparable among sites and species. Novak et al. (2013b) applied a standardization procedure to IADFs in *P. halepensis* to remove the effect of age, whereas Campelo et al. (2015) adopted a different method in *P. pinaster* to remove the effect of tree-ring width from IADF series. Here, a new approach was used to remove the effect of tree-ring age from IADF series across several species. This approach could be extended to other species across different geographical ranges and environments to facilitate the comparison of results and to gain univocal information.

IADF Frequency Geographical Pattern and Tree-Ring Width Relationship

The map of mean detrended frequencies of IADFs of the studied sites helped to show the spatial distribution of frequency among species and geographical location, pointing out a potential climatic influence. The highest values of IADF frequency were found in the sites located at longitudes where Mediterranean

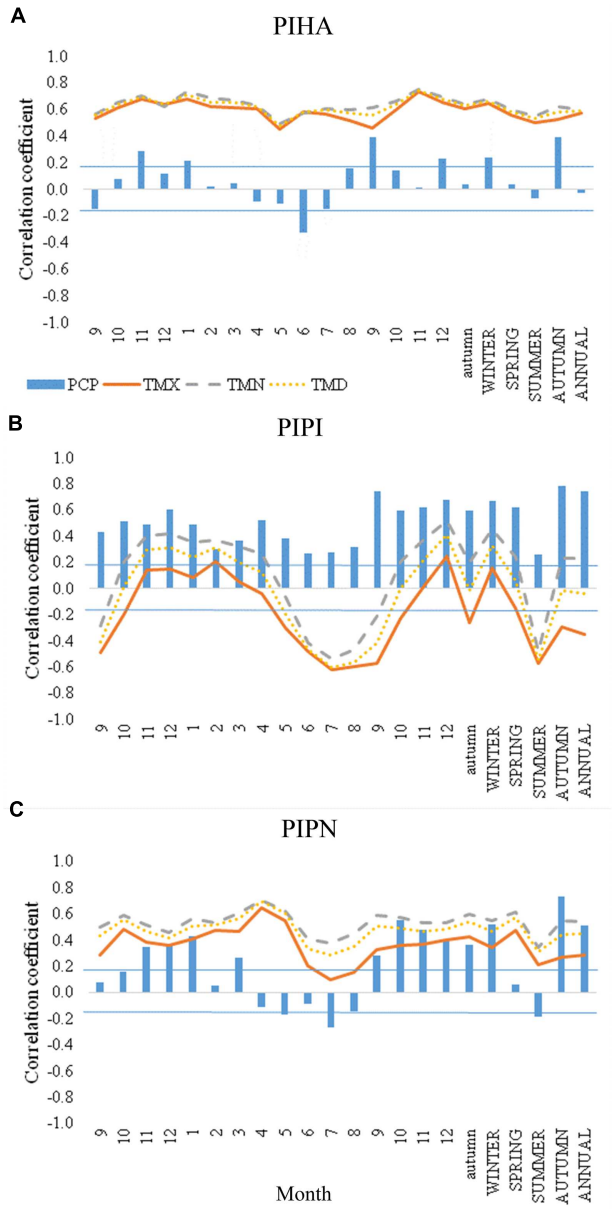


FIGURE 5 | Correlation coefficient values between IADF frequency, total precipitation (PCP) and maximum (TMX), minimum (TMN), and mean (TMD) monthly temperature for the three species [(A) *Pinus halepensis* (PIHA), (B) *Pinus pinaster* (PIPI), and (C) *Pinus pinea* (PIPN)]. IADF frequency is correlated with climate data at monthly and seasonal scale from September of previous year to December of the current year, and at annual scale. First numbers from 9 to 12 to the left of the graphs and lowercase letters refer to the previous year. Blue horizontal lines indicate the limits of the correlation coefficient (±0.196) for significance at $p < 0.05$ ($n > 100$).

climate is affected by oceanic influences, as Portugal and north-west Spain. The lowest values were found in the sites located in eastern Spain, where the climate ranges from Mediterranean to semiarid. High values of IADF frequency were also found for sites with warm Mediterranean climate as the ones located in Italy, southern Portugal and south-central Spain (Peel et al., 2007). The inter-specific analysis highlighted the relationship between IADF frequency and growth rate, and its relation with climate: *P. pinaster* was the species with the highest frequency of IADFs and the widest tree rings at the same time, growing in sites with the highest mean precipitations throughout the year and mild winter conditions. On the opposite, the lowest frequencies of IADFs were recorded in tree rings of *P. halepensis* that was also the species with the highest percentage of narrow rings, growing in sites characterized by the lowest values of mean precipitation throughout the year. In all the species studied, the relationship between IADF frequency and tree-ring width showed that IADFs tend to be more frequent in wide but not in the widest rings. This result is mainly in agreement with the recent finding of Carvalho et al. (2015) who suggests that the formation of IADFs in latewood of *P. pinaster* is predisposed by higher rates of cell production in spring which, in turn, increases the number of cells under enlargement after the summer drought, leading to the formation of wider rings. This could explain the case of *P. pinaster* in Portugal and north-west Spain, and *P. pinea* in Italy, under temperate and Mediterranean conditions, respectively, showing the highest IADF frequencies and the widest tree rings. Regarding the reason why IADF frequency decreases or remains stable in extreme wider tree rings, we hypothesize that extremely wider rings are usually formed during years with favorable conditions for tree growth throughout the growing season (Fritts, 1966) without fluctuations in environmental conditions, which is considered to be the main triggering factor of IADF formation (Carvalho et al., 2015). On the opposite, the low frequency of IADFs in narrow tree-rings may be attributed to particularly unfavorable conditions during the growing season (Fritts, 1966; Zubizarreta-Gerendiain et al., 2012). In this case, trees may not have enough reserves to allow the resumption of cambial activity as a response to favorable climatic conditions after the summer drought. This could explain the situation mainly found in *P. halepensis* and *P. pinea* sites in eastern Spain under a semi-arid Mediterranean climate (Peel et al., 2007), showing the lowest values of IADF frequency.

Climatic Signal in IADF

The climate correlations allowed us to standardize the large amount of data from the three species growing under different microclimatic conditions and in populations with different structures, with the aim of analyzing common regional patterns over local ones. A common large-scale climatic factor driving IADF formation was autumn precipitation, with high values of correlation coefficients for all the analyzed species. These results agree with the hypothesis that the formation of IADFs in Mediterranean pines is mainly triggered by the resumption of cambial activity in response to the return of favorable conditions, such as autumn precipitation after summer drought (Campelo et al., 2007; de Luis et al., 2007, 2011a,b; Camarero et al., 2010; Novak et al., 2013a,b; Hetzer et al., 2014; Carvalho et al., 2015). A decrease in precipitation during summer, associated with an increase in temperatures, may influence cell division and expansion, slowing down cambial activity (Antonova and Stasova, 1997; Deslauriers and Morin, 2005; Vieira et al.,

2014). Tracheids with narrow lumen and thick wall (latewood or latewood-like cells) are formed in response to low cell turgor during summer drought (Domec and Gartner, 2002). When water availability increases after autumn precipitation, differentiating cells can promptly re-acquire enough pressure (Wimmer et al., 2000; Abe et al., 2003; Rossi et al., 2009) for the enhancement of lumen enlargement (Wimmer et al., 2000), leading to earlywood-like cells (Carvalho et al., 2015; Vieira et al., 2015).

Late summer/autumn precipitation preceded by a drier period seemed to be the common triggering factor of IADFs in the three species. However, the species appear to be differently predisposed to the formation of IADFs depending on the geographic influence by peculiar climatic conditions experienced throughout the year.

Temperature seemed to play an important role for IADF formation in *P. halepensis* and *P. pinea* as confirmed by positive correlations between temperature and IADF frequency throughout the year. Favorable conditions of growth with high temperatures throughout the year could be a predisposing factor for the formation of IADFs in these two species, allowing them to efficiently react to seasonal fluctuations of precipitation. Moreover, temperatures suitable for growth throughout the year can induce a longer growing season, resulting in wider rings that are generally more prone to form IADFs (Deslauriers et al., 2008; Campelo et al., 2015). Regarding *P. pinaster*, living in the wetter sites of our network, precipitation seems to have a major role for IADF formation, as suggested by the positive correlations between precipitation and IADF frequency throughout the year. Furthermore, a mild dry summer was found to be a significant factor leading to the formation of IADFs in this species, as showed by the highly significant negative correlations of IADF frequency with summer-early autumn temperature. Favorable conditions for growth with wet conditions at the beginning of the growing season could facilitate *P. pinaster* to have a second period of cambial activity during autumn, after the summer drought, as suggested by Pacheco et al. (2016) for Spanish juniper. On the contrary, severe drought periods may prevent the formation of IADFs leading *P. pinaster* to an earlier stop of the growing season, unable to resume cambial activity in response to increased water availability (Vieira et al., 2014).

The methodological approach used in this study helped to link IADF frequency to climate conditions on a regional scale, but does not solve the question of the species-specific nature of their formation. There is evidence of a geographical/environmental gradient for *P. halepensis*, with more frequent IADFs in coastal than in inland or high elevated sites (Novak et al., 2013b). In our analysis, this trend was evidenced in *P. halepensis* but not in the other two pine species. The expansion of the network, especially by addition of new inland sites, could be useful to gain further insight on species-specific microclimate associations in IADF formation.

However, distribution ranges of the three species do not completely overlap, with wide geographical areas where the species do not coexist, making the comparison between species growing under the same weather conditions difficult. Thus, further research is needed using complementary approaches (including modeling) in order to disentangle the species-specific nature of IADF formation from the dependence on climate at a regional level.

Furthermore, several studies have shown the importance of the position of IADFs within the tree rings since it could reflect different climatic triggering factors of IADF formation and could also influence their frequency (Campelo et al., 2007; Battipaglia et al., 2010; De Micco et al., 2012). In the present work, we did not evaluate the relative position of IADFs within rings, since their identification is not straightforward as different methodological approaches exist (Campelo et al., 2007; De Micco et al., 2012, 2014). However, this kind of information is valuable to better understand the temporal link between IADFs and environmental factors, thus further improvements are needed to include this information in this type of analysis on a broad geographical scale.

CONCLUSION

To date, a few studies have analyzed climatic influences on IADF formation across environmental gradients, and, to our knowledge, there is no study that combines spatial and temporal variations of IADF frequency and climate across a wide geographical range. Our results showed that tree-ring age has to be taken into account when analyzing IADFs-climate relationships because it plays an important role in IADF formation in Mediterranean pines.

A common interval of tree-ring width presenting the highest frequency of IADFs was found for the three species, with a more frequent formation of IADFs in tree rings moderately wide. Moreover, a common large-scale climatic factor driving IADF formation was found in autumn precipitation, demonstrating the potential of IADFs for climatic reconstructions. IADF formation was found to be lower in species living under Mediterranean to semi-arid conditions, where the frequency of narrow rings is higher (e.g., *P. halepensis* in eastern Spain). However, the highest frequency of IADF formation was found in species living in temperate sites with oceanic influence, where wetter conditions throughout the year associated with a moderately dry period in summer lead to the formation of wider tree rings (e.g., *P. pinaster* in Portugal and in north-west Spain). A proxy record of the intra-annual plastic response of wood traits on a large scale could add insights on global change studies as highlighted for anatomical parameters by Fonti et al. (2010). IADFs could be analyzed in a large tree-ring network and used as efficient indicators to predict the plastic adjustment of tree species to changing environmental conditions, especially in the climate hotspot of the Mediterranean ecosystems. Their occurrence in several Mediterranean species, particularly conifers as *Pinus* spp. enabled this pioneering study of IADFs on a wide network which might be further expanded to the entire Mediterranean Basin.

AUTHOR CONTRIBUTIONS

EZ, ML, and GB gave a substantial contribution to the conception and design of the study. All authors contributed to the supply of

data for the network. ML and EZ were in charge for statistical analyses. EZ, VM, FC, and GB contributed to data analysis. EZ, ML, FC, VM, and GB gave substantial contribution to the interpretation of data. EZ wrote the main part of the manuscript. ML, FC, VM, GB, and EZ performed the critical revision of the work. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

Collection of datasets used for this work was supported by the projects: ELENA (CGL2012-31668) and CGL2015-69985-R funded by the Spanish Science and Innovation Ministry (MICINN) and FEDER funds; PGIDIT06PXIB502262PR funded by Xunta de Galicia; POCI/CLI/58680/2004 and PTDC/AAC-AMB/111675/2009 funded the Portuguese Foundation for Science and Technology (FCT) and the European Union (POCI 2010).

The work of EZ was partially funded by COST Action STREeSS (COST-FP1106) through a Short-Term Scientific Mission (STSM).

ACKNOWLEDGMENTS

This article is based upon work from COST Action FP1106 STReESS, supported by COST (European Cooperation in Science and Technology). We thank the numerous collaborators for field and laboratory assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00579

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.10 SUPPLEMENTARY MATERIAL

Tab. S1 Details of each analyzed site of the network. Site numbers related to IADF chronologies newly processed in this study are marked with an asterisk.

	Site number	Site	Site code	Country	Altitude	Longitude	Latitude	N of trees	N of samples	Starting year	End year
Pinus halepensis											
	1	Alcoy (Penaguila)	ALC	Spain	674	-0.36	38.68	15	30	1852	2000
	2	Alloza	ALL	Spain	595	-0.57	40.98	15	31	1888	2006
	3	Ayerbe	AYE	Spain	924	-0.84	42.32	17	33	1946	2006
	4	Biar	BIA	Spain	806	-0.77	38.62	15	29	1927	2000
	5	Alcubierre	CAP	Spain	738	-0.50	41.75	22	36	1800	2006
	6	Caspe	CAS	Spain	166	0.07	41.29	15	28	1845	2006
	7	Cartagena	CAT	Spain	116	-1.01	37.61	15	27	1915	2007
	8	Chiprana	CHI	Spain	160	-0.09	41.24	10	10	1900	2003
	9	Crevillente	CRE	Spain	285	-0.77	38.29	12	43	1832	2000
	10	Daroca	DAR	Spain	937	-1.41	41.14	14	28	1934	2006
	11	Ejea-Bardenas	EBA	Spain	365	-1.40	42.17	9	9	1909	2003

	12	Estopinian del Castillo	EST	Spain	502	0.61	41.97	14	27	1964	2006
	13	Font de la Figuera	FHI	Spain	680	-0.93	38.83	14	28	1946	2011
	14	Alcoy (Font Roja)	FNT	Spain	1022	-0.54	38.67	14	24	1864	2005
	15	Fraga	FRA	Spain	340	0.32	41.47	14	29	1844	2006
	16	Fuensanta	FUE	Spain	138	-1.12	37.94	14	26	1902	2007
	17	Gilet (Urbanizacion La Pinada)	GI2	Spain	140	-0.31	39.67	14	26	1899	2011
	18	Gilet (SantEspirit)	GIL	Spain	175	-0.35	39.67	14	26	1892	2006
	19	El Grado	GRA	Spain	168	0.20	42.16	15	30	1946	2006
	20	Guardamar	GUA	Spain	15	-0.65	38.10	11	39	1912	2000
	21	Jalance	JAL	Spain	571	-1.15	39.19	22	49	1863	2004
	22	Javea	JAV	Spain	96	0.19	38.73	15	60	1924	2000
	23	MaigmoNorte	MAN	Spain	845	-0.64	38.52	35	70	1867	2009
	24	MaigmoSur	MAS	Spain	762	-0.60	38.50	23	39	1901	2009
	25*	Montes de malaga	MDM	Spain	135	-4.42	36.76	13	26	1956	2012

	26	Montanejos	MON	Spain	569	-0.54	40.06	16	31	1955	2001
	27	Oliete	OLI	Spain	530	-0.69	40.99	15	28	1960	2006
	28	Oropesa	ORO	Spain	1	0.12	40.06	15	30	1921	2003
	29	Mallorca (Binissalem)	PA1	Spain	120	2.86	39.69	13	26	1914	2011
	30	Mallorca (Caimari)	PA2	Spain	386	2.89	39.79	13	26	1888	2011
	31	Mallorca (Cap Salines)	PA3	Spain	14	3.05	39.27	12	23	1890	2011
	32*	Cazorla - Puerta de Segura	PSC	Spain	1030	-2.72	38.36	15	28	1944	2012
	33	Puerto de Ragudo	RAG	Spain	959	-0.65	39.97	15	29	1964	2011
	34	Requena	REQ	Spain	721	-1.20	39.47	15	31	1789	2003
	35*	Sierra de Huetor	SDH	Spain	1120	-3.54	37.27	15	30	1965	2012
	36	Sierra Espuña	SES	Spain	846	-1.52	37.86	16	29	1894	2007
	37	Villanueva de Gallego	VLL	Spain	452	-0.91	41.88	15	29	1878	2006
	38	Zorita	ZOR	Spain	857	-0.11	40.74	15	30	1832	2001

Pinus pinea											
	1	Alcácer do Sal	ASPI	Portugal	160	-8.52	38.10	25	50	1896	2003
	2	Mértola	BEME	Portugal	190	-7.49	37.71	7	14	1917	2002
	3	Serpa	BESE	Portugal	210	-7.50	37.90	5	10	1929	2002
	4*	Castelporziano	CP	Italy	10	12.40	41.74	21	21	1897	2003
	5	Daroca	DAR	Spain	937	-1.41	41.14	15	31	1894	2007
	6*	Duna Feniglia	DF	Italy	10	11.22	42.44	14	14	1924	2003
	7	Portel	EVPO	Portugal	270	-7.73	38.25	5	10	1939	2002
	8	Guardamar	GUA	Spain	15	-0.65	38.10	14	27	1914	2003
	9*	Montes de Malaga	MDM	Spain	135	-4.42	36.76	9	17	1888	2012
	10*	Rubielos Altos	RBA	Spain	800	-2.05	39.48	12	23	1930	2012
Pinus pinaster											
	1	Monte Aloia	ALO	Spain	530	-8.68	42.08	21	21	1967	2006
	2	A Barrela	BAR	Spain	620	-7.85	42.53	23	23	1967	2006

	3	A Capelada	CAP	Spain	340	-7.98	43.67	22	22	1967	2006
	4	Isla de Cortegada	COR	Spain	20	8.78	42.62	22	22	1967	2004
	5*	Despeñaperros	DES	Spain	600	-3.51	38.37	10	20	1964	2012
	6	Monte Insua	INS	Spain	10	-9.15	43.13	14	14	1967	2006
	7	Leiria	LEMG	Portugal	33	-8.99	39.76	40	80	1818	2006
	8	Marco da Curra	MCU	Spain	590	-7.88	43.37	19	19	1967	2006
	9	Muros	MUR	Spain	155	-9.07	42.80	21	21	1967	2006
	10*	Isola d'Elba	PPFM	Italy	420	10.20	42.77	8	8	1964	2007
	11*	Isola d'Elba	PPTP	Italy	460	10.18	42.77	9	9	1962	2007
	12*	Cazorla – Puerta de Segura	PSC	Spain	1050	-2.72	38.36	10	20	1870	2012
	13*	Sierra de Huetor	SDH	Spain	1300	-3.49	37.25	11	21	1942	2012
	14*	Serra de Estrela	SEPP	Portugal	1050	-7.55	40.38	26	56	1907	2010
	15	Geres	SG	Portugal	1500	-8.00	42.00	18	36	1907	2007

	16	Tocha	TCHA	Portugal	16	-8.80	40.33	60	120	1953	2008
	17	Trabada	TRA	Spain	640	-7.22	43.42	23	23	1967	2006
	18	Verin	VER	Spain	855	-7.55	42.07	23	23	1967	2006
	19	Vigo	VIG	Spain	365	-8.65	42.20	19	19	1967	2006

Tab. S2 Number of rings, number of IADFs, mean raw and age-detrended frequency of the sites of the network.

	Site number	Site code	N of rings analyzed	N of rings with IADFs	Raw frequency (%)	Age-detrended frequency
Pinus halepensis						
	1	ALC	3760	32	6.23	0.07
	2	ALL	2878	110	2.32	0.25
	3	AYE	1377	32	0.33	0.11
	4	BIA	1771	207	3.82	0.58
	5	CAP	2371	37	17.15	0.07
	6	CAS	3514	33	12.20	0.07
	7	CAT	2039	161	4.48	0.39
	8	CHI	940	36	1.56	0.22
	9	CRE	3456	213	0.94	0.34
	10	DAR	991	170	3.83	0.84
	11	EBA	571	12	2.10	0.10
	12	EST	1044	65	1.73	0.30
	13	FHI	1498	135	1.54	0.44
	14	FNT	1936	78	4.74	0.22
	15	FRA	3702	64	5.92	0.12
	16	FUE	2094	539	0.85	1.39
	17	GI2	1737	321	11.69	1.01
	18	GIL	2235	362	9.01	0.89
	19	GRA	1505	5	4.03	0.02
	20	GUA	2377	383	6.59	0.87
	21	JAL	4308	284	5.01	0.39

	22	JAV	3433	1197	8.43	1.69
	23	MAN	4932	247	2.33	0.29
	24	MAS	2692	227	6.16	0.43
	25	MDM	1303	295	18.48	1.09
	26	MON	1223	58	16.20	0.22
	27	OLI	1049	128	16.11	0.57
	28	ORO	2022	332	34.87	0.81
	29	PA1	2187	427	16.42	1.10
	30	PA2	2031	336	7.90	0.85
	31	PA3	2336	155	25.74	0.40
	32	PSC	1644	92	7.98	0.27
	33	RAG	1266	75	5.60	0.29
	34	REQ	3858	90	22.64	0.30
	35	SDH	1180	20	1.69	0.09
	36	SES	2670	213	19.52	0.54
	37	VLL	2597	40	16.54	0.08
	38	ZOR	2725	122	6.64	0.29
Pinus pinea						
	1	ASPI	2854	771	27.01	1.57
	2	BEME	799	428	53.57	2.77
	3	BESE	578	221	38.24	1.96
	4	CP	2174	623	28.66	1.78
	5	DAR	2962	308	10.40	0.62
	6	DF	956	483	50.52	2.61
	7	EVPO	533	217	40.71	1.99
	8	GUA	2114	319	15.09	0.88
	9	MDM	1814	250	13.78	0.94

	10	RBA	1346	29	2.15	0.12
Pinus pinaster						
	1	ALO	663	413	62.29	3.05
	2	BAR	884	680	76.92	3.67
	3	CAP	852	375	44.01	2.13
	4	COR	825	769	93.21	4.50
	5	DES	875	302	34.51	1.61
	6	INS	484	240	49.59	2.38
	7	LEMG	5213	1790	34.34	2.45
	8	MCU	760	355	46.71	2.25
	9	MUR	839	579	69.01	3.38
	10	PPFM	327	206	63.00	2.97
	11	PPTP	338	112	33.14	1.64
	12	PSC	1970	133	6.75	0.34
	13	SDH	1341	94	7.01	0.38
	14	SEPP	4711	2292	48.65	2.92
	15	SG	2756	1616	58.64	3.39
	16	TCHA	5634	2283	40.52	1.92
	17	TRA	915	228	24.92	1.19
	18	VER	885	237	26.78	1.28
	19	VIG	742	461	62.13	2.99

3. Intra-Annual Density Fluctuations in tree rings of *Quercus ilex* L. and *Pinus pinea* L. as functional indicators of species-specific responses to intra-annual climate variability

3.1 Abstract

Investigating plant reactions to past climatic variability is a valuable issue, particularly to understand how different species will cope with forecasted changes. Mixed forests of *Quercus ilex* L. and *Pinus pinea* L. are widely found throughout the Mediterranean Basin as representative of two coexisting functional types: evergreen-sclerophyllous drought-resistant species and Mediterranean-adapted drought-avoidant conifers. Their contrasting physiological strategies to cope with water deficit influence all the processes regulating their growth such as wood formation, leading to peculiar tree-ring anatomical features such as different types of intra-annual density fluctuations (IADFs). IADFs are abrupt changes in density within a tree ring, and are frequently found in Mediterranean species as a response to seasonal climate changes. In this study, we characterised the anatomical traits and composition of carbon and oxygen stable isotopes in IADFs occurring in tree rings of *Q. ilex* and *P. pinea* trees co-existing at a same site in Southern Italy, in order to link their xylem hydraulic properties with the related physiological mechanisms. Furthermore, the relationship between IADF occurrence and seasonal mean temperature and total precipitation was investigated, with the aim of assessing whether they can be used as indicators of species-specific responses to intra-annual climate fluctuations. The used approach allowed the anatomical and isotopical characterization of IADFs in *Q. ilex* and *P. pinea* coetaneous trees growing at the same Mediterranean stand. Results lead to the hypothesis that IADF period of formation is during autumn months for both species. The influence of climate on IADF occurrence was found to be an indicator of species-specific response to climate: an increased stomatal conductance associated to the formation of a safer wood was found in *Q. ilex*, while a tighter stomatal control associated to a more efficient wood at IADF level occurred in *P. pinea*. Moreover,

the assessment of the influence of climate on IADF occurrence helped to forecast that, with rising temperatures, *Q. ilex* would form less IADFs compared to *P. pinea*. Other study cases are desirable to assess the suggested forecasts and to link the plasticity of the species to form IADFs with their effective adaptive capability to compete for resources and to explain how it may influence future population development.

3.2 Introduction

Mediterranean forests will be subjected to the forecasted increase in climate variability with frequent extreme weather events, such as extended drought periods (Lindner et al., 2010). In Mediterranean areas, a warming trend has been recorded from 1990 to recent years (Hansen et al., 2006, 2010; IPCC, 2017), with no concomitant increase in precipitation which were also shifted to winter months; therefore, in such areas a sensitive response of trees to temperature rise could be expected (Loustau et al., 2005). Sensitivity and vulnerability of plant populations to climate are site-specific and depend on the adaptive capacity of the species (Lindner and Calama, 2013). Even drought-adapted plants are influenced by severe water shortage (Rambal et al., 2003), and ecophysiological differences between coexisting species may influence competition and thus be decisive for future population development.

Mixed forests of *Q. ilex* L. and *P. pinea* L. are extensively found throughout the Mediterranean Basin, being representative of two coexisting functional types: evergreen sclerophyllous drought resistant species and Mediterranean-adapted drought avoidant conifer (Terradas, 1999). *Q. ilex* is a shade-tolerant tree, becoming dominant in late successional stages; it is deep rooted and has a great capacity to mantain high stomata conductance during dry periods (Barbero et al., 1992). On the contrary, *P. pinea* has a shallow root system and reduces water flow during aridity as a drought-avoidant species, being able to recover when water becomes available again (Mayoral et al., 2015; Zalloni et al., 2016). The contrasting physiological strategies adopted by *Q. ilex* and *P. pinea* to

cope with water stress indicate different sensitivity to temperature and to water availability and influence all the processes regulating their growth. Therefore, wood formation would be ultimately affected differently in the two species thus leading to peculiar tree-ring anatomical features such as intra-annual density fluctuations (IADFs). IADFs are abrupt changes in density within a tree ring, and are frequently found in Mediterranean species as a response to seasonal climate changes (Cherubini et al., 2003, De Micco et al., 2016a). The physiological bases leading to IADFs could be extrapolated by the analysis of functional wood traits and $\delta^{13}C$ and $\delta^{18}O$ of tree rings at an intra-annual level (Battipaglia et al., 2010, 2014; De Micco et al., 2007, 2012). In several cases, in *P. pinea* and other Mediterranean conifers IADF occurrence in Mediterranean species has been related to the alternation of drought in either summer or autumn followed by a wet period (Bogino and Bravo, 2009; Camarero et al., 2010; Campelo et al., 2007b, 2013, 2015; Carvalho et al., 2015; de Luis et al., 2007, 2011; De Micco et al., 2007; DeSoto et al., 2011; Nabais et al., 2014; Novak et al., 2013a,b; Olivar et al., 2012, 2015; Rozas et al., 2011; Vieira et al., 2009, 2010, 2015; Zalloni et al., 2016). A similar relation has been reported in *Q. ilex* and other angiosperms (Battipaglia et al., 2010, 2014; Campelo et al., 2007a; Cherubini et al., 2003; Copenheaver C.A., 2010; De Micco and Aronne, 2009; Zhang and Romane, 1991). To the best of our knowledge, IADFs in tree rings of *Q. ilex* and *P. pinea* have not been characterized yet in terms of anatomical and isotopical traits, although IADF frequency was deeply analyzed in *P. pinea* as shown in the previous chapter. Quantifying anatomical traits of IADFs together with their stable isotope composition is useful to link xylem hydraulic properties with physiological mechanisms related to their formation. $^{13}C/^{12}C$ and the $^{18}O/^{16}O$ intra-annual ratios help to reconstruct CO_2 assimilation of the plant at a sub-annual level, reflecting photosynthetic rate and stomatal conductance, thus plant water-use efficiency and response to environmental stresses such as drought (Farquhar and Richards, 1984; Leavitt, 1993; Matzner et al., 2001; Panek and Goldstein, 2001; Ponton et al., 2001; Scheidegger et al., 2000). Quantitative wood anatomy and carbon stable isotope ratio in the IADFs of *P. pinaster* Aiton. were proved to be indicative of a drought period (De Micco et al., 2007), while they helped to distinguish

between two different types of IADFs in *Arbutus unedo* L., which were induced by drought deficit or by wet conditions depending on different local microclimates (Battipaglia et al., 2010). In this study we characterised anatomical traits and carbon and oxygen stable isotope composition in IADFs occurring in tree rings of *Q. ilex* and *P. pinea* trees co-existing at a same site. We investigated the relationship between their occurrence and seasonal mean temperature and total precipitation with the aim of assessing whether they can be used as indicators of species-specific responses to intra-annual climate fluctuations. Respectively, we hypothesise that high IADF frequency occurs in the same years for the two species, driven by temperature and precipitation of the hottest months and of the rainy season, with *P. pinea* more influenced by high temperature than *Q. ilex*, reflecting its drought-avoidant strategy. Furthermore, we hypothesise a different carbon and oxygen isotopic composition in the tree rings of the two species. In particular, we expect that IADF wood of *Q. ilex* is less enriched in $\delta^{13}C$ and $\delta^{18}O$ compared to the other intra-ring regions while *P.pinea* IADF should be more enriched than the rest of the ring, reflecting the contrasting physiological strategies of the two species to cope with dry periods. Finally, we hypothesise that the anatomical functional traits of IADFs are the result of the different balance between efficiency and safety in the two species. This approach would be useful to add valuable information on *Q. ilex* and *P. pinea* mixed forests responses to drought and their ecological role for Mediterranean forest dynamics.

3.3 Materials and Methods

3.3.1 Study site

The study sites are located in the "Tirone Alto-Vesuvio" Forest State Reserve, on the southwest slope of the Somma-Vesuvio volcanic complex (40°48'46.5"N, 14°24'10.6"E, 505 m a.s.l.), at about 15 km SE of Naples (Southern Italy) (Fig. 1). The site is covered by *Quercus ilex* L. and *Pinus pinea* L. with an understory consisting of young *Q. ilex* plants, with the sporadic presence of

Robinia pseudoacacia L., a non-native invasive species. Mean diameter and height of dominant trees are 37.55±1.49 cm and 17.53±0.48 m (mean value±SE) for *Q. ilex*, while 52.5±1.72 cm and 16.6±0.45 m for *P. pinea*. Stand density is of 13000 tree/ha, with 10000 *Q. ilex* trees and 3000 *P. pinea* trees per hectare; stand canopy cover is of 1.69 (leaf area index - LAI), while stand slope is almost null. Temperature and precipitation data from the nearest meteorological stations were fragmented and didn't cover all the period of interest; therefore they were interpolated and compared to the CRU TS3.23 gridded dataset at 0.5° resolution data for the period 1951-2014 (Harris et al., 2014). Since the correlation between the two data series was significant (supplementary material, Fig. S1, Table S1), we considered the climate data from the CRU to be representative for our study sites. The climate is Mediterranean with mild winter and hot summer with a mean annual temperature of 16.1°C and precipitation mainly concentrated in autumn and winter and at the beginning of spring, with an average of 766 mm per year. The drought period lasts from July to August, with a maximum monthly temperature of 32.6 °C and a minimum precipitation of 0 mm. The higher amount of precipitation is reached in November, with a maximum monthly value of 252.5 mm (Fig. 1).

3.3.2 Soil analysis

Water content, available water capacity and water holding capacity were determined by taking 6 samples random within the site and following standard procedures (USDA, Natural Resources Conservation Service, National Soil Survey Center, 1996). Undisturbed soil was collected with a PVC 10-cm cylinder with known weight filled up to the top. Each cylinder was sealed at the lower end with gauze and brought to the lab in a plastic bag without overturn it. The samples were then weighted (fresh weight) and placed in a few inches of distilled water for 48 hours to allow the water to rise by capillarity. Then, after the loss of the excess of water by percolation, samples were weighted to determine field capacity (the optimal condition of soil water content with saturated

micropores). The soil of each sample was finally moved into aluminum bowls and placed in a stove at 105°C. Dry weight was determined when the samples reached a constant weight after drying.

3.3.3 Tree-ring width analysis

Twenty dominant *Q. ilex* trees and twenty dominant *P. pinea* trees were sampled, taking two increment cores per tree at breast height (1.3 m) with a 0.5 cm diameter increment borer (Haglöf Sweden) following standard methods (Schweingruber, 1988). The increment cores were air dried, mounted on wooden supports and sanded with progressively finer grades of sandpaper (up to 600 grit). Tree rings were visually crossdated and tree-ring width was measured with a resolution of 0.01 mm using a Leica MS5 stereoscope (Leica Microsystems, Germany), fitted with a LINTAB measuring system (Frank Rinn, Heidelberg, Germany), and analyzed with the TSAP-Win software. Cross-dating accuracy of the individual series was checked using TSAP-Win and the software COFECHA, which performs the segmented time-series correlation technique (Holmes, 1983); mean chronologies of the sites and chronology quality assessment were implemented with the software R using the Dendrochronology Program Library (dplR; Bunn, 2008, 2010). Chronology quality was assessed calculating the total correlation between trees (R_{bt}) as a measure of common signal strenght, and the espressed population signal (EPS) which defines how well a chronology based on a finite number of trees approximates the theoretical true population chronology based on the threshold value of 0.85 (Wigley et al., 1984). Age and low-frequency growth trends were removed in order to perform climate correlations by fitting a smoothing spline curve with a 50% frequency response cutoff and a rigidity of the 67% of series lenght to the individual series (Cook and Peters, 1981). The observed values were divided by the estimated spline curves to obtain indexes, and an autoregressive model was performed to remove the first-order autocorrelation. Mean detrended chronologies of the sites were then obtained by averaging the index series using a biweight robust mean to reduce the influence of outliers. On detrended series, mean sensitivity (MS) was calculated

indicating the mean percentage change from each measured yearly ring value to the next (Fritts, 1976).

3.3.4 IADF identification and frequency

The annual IADF presence was detected within the rings of the correctly dated cores under a stereomicroscope, following the identification given by Campelo et al. (2007a) for *Q. ilex* and by Campelo et al. (2007b) for *P. pinea*. Relative annual IADF frequency chronologies of the sites were calculated as the ratio between the number of cores with IADFs and the total number of cores per each year. Stabilized annual IADF frequency chronologies were then calculated according to Osborn et al. (1997) as $f = Fn^{0.5}$, where F is the relative IADF frequency value and n is the total number of cores for each year, in order to stabilize the variance and to overcome the issue of the changing sample depth over time. The age trend of the chronologies was removed by dividing the best fitted estimated polynomial curves for age-based chronologies, built by aligning the individual series of IADF presence/absence by cambial age (considering pith-offset estimations), from the original observed values.

3.3.5 Quantitative wood anatomy analysis

Anatomical traits were analyzed in all tree rings from 5 cores in both *Q. ilex* and *P. pinea* (304 and 289 tree rings, respectively). More specifically, in *Q. ilex*, tree-ring microphotographs were taken from the observed cores under a dissection microscope (SZX16, Olympus, Germany), equipped with a XM10 camera (Olympus), after filling the vessels with chalk (Gärtner & Schweingruber, 2013). In *P. pinea*, semi-thin (20 μm) sections were cut with a GSL1 microtome (Gärtner et al., 2014), dehydrated with 70% ethanol, mounted on slides with immersion oil for fluorescence microscopy, and observed under an epi-fluorescence microscope (BX60, Olympus, Germany)

equipped with a Camedia 4040 camera (Olympus). For epifluorescence microscopy, a mercury lamp with a band pass filter BP 330-385, dichromatic mirror > 400 nm and a barrier filter > 420 nm was used to detect autofluorescence of lignin and phenolic compounds (Ruzin, 1999; Wu et al., 1992). The images were analyzed with the software AnalySIS 3.2 (Olympus) to quantify tree-ring anatomical features. Vessel lumen area was quantified *in continuum* from the beginning (earlywood) to the end (latewood) of each ring along a transect between two rays for *Q. ilex* (with an area between 378.87 ÷ 9944.16 $10^3\mu m^2$), and along three lines of cells for *P. pinea*, following the standardized centre of gravity method (De Micco et al., 2014). In brief, vessel distance from the beginning of the ring was standardized by dividing vessel lumen centres of gravity recorded by the software by the total ring width, and multiplying by 100, with the whole ring width being considered equal to 100%. As a result, each vessel was characterized by two coordinates: Y as the lumen area in $10^3\mu m^2$ for *Q. ilex* and in μm^2 for *P. pinea,* and X as the distance from the beginning of the ring, expressed as a percentage. The so-ordered data were pooled together for rings with and without IADFs for each species and plotted into dispersion graphs. Polynomial curves were then fitted to the data points to explore vessel area intra-annual growth trends. Furthermore, each ring along the same transects was divided in four equal regions (with each region corrisponding to the 25% of total ring width) for the quantification of wood density and vessel frequency parameters (vessel frequency was measured only for *Q. ilex* samples). Wood density was quantified as the percentage of cell walls over total xylem area for each region (De Micco et al., 2016b) while vessel frequency was calculated as the number of vessels per $10^3\mu m^2$, determined by counting vessels in a known area according to Wheeler et al. (1989). One-way ANOVA was performed with the SPSS® statistical package (SPSS Inc., Chicago, IL, USA) to investigate the differences between regions within rings and between the same region of rings with and without IADFs.

3.3.6 Stable isotope analysis

The same trees selected for anatomical analysis were used for carbon and oxygen stable isotope measurement in the two species. Isotope analysis were conducted over the period 1985-2005 which corresponded to a wide variable range of IADF frequency values. Age-related trends were also avoided. Tree rings were manually split into intra-annual sections with a scalpel under a dissection microscope, and the derived samples of the five cores per species and per site were then pooled together in order to maximize sample size, leaving them separate only for the years 1985, 1995 and 2005 in order to verify inter-tree variability. Following quantitative wood anatomy observations and previous studies (Battipaglia et al., 2010; De Micco et al., 2007), tree rings with IADFs were split into three sections (from pith to bark: earlywood, latewood and IADF) for *Q. ilex*, while into four (from pith to bark: earlywood, early-latewood, IADF and latewood) for *P. pinea* (supplementary material, Fig. S2). Instead, tree rings without IADFs were split into two sections (earlywood and latewood) for both species. Whole wood was used for the analysis, without any chemical pre-treatment. The question on whether cellulose extraction from wood samples is needed or not to investigate environmental effects on stable isotopes is still open (Gessler et al., 2014). However, compareble results have been found using either whole wood or cellulose (Barbour et al., 2001; Borella et al., 1998; Korol et al., 1999; Loader et al., 2003; Verheyden et al., 2005; Warren et al., 2001; Weigt et al., 2015). Thus we decided to proceed on whole wood, also to have enough wood material for isotope analyses also in the case of very narrow rings. The samples were then milled with a centrifugal mill, weighted in silver capsules (aliquots of 0.8÷1.0 mg) and pyrolyzed at 1450°C (PYRO-cube, Elementar, Hanau, Germany) in order to determine the intra-annual $\delta^{13}C$ and $\delta^{18}O$ values of the obtained CO by a Delta Plus XP isotope ratio mass spectrometer (ThermoFinnigan MAT, Bremen, Germany) via a pyrolysis unit by a ConFlo III interface (ThermoFinnigan MAT). A correction of the resulted $\delta^{13}C$ values was made by measuring a subset of samples that covered the whole range of the expected $\delta^{13}C$ values via oxygen combustion, since the $\delta^{13}C$ signal obtained by pyrolysis is considered to be dampened because of "memory effects",

compared to the most commonly used one obtained by oxygen combustion (Woodley et al., 2012). $\delta^{13}C$ values of the subset were measured via oxygen combustion with an EA1110 elemental analyzer (CE Instruments, Milan, Italy) coupled to a Delta-S isotope ratio mass spectrometer (ThermoFinnigan MAT). The correction used for the pyrolysis $\delta^{13}C$ data was the following: $\delta^{13}C_{corr} = 1.2526 \times \delta^{13}C_{pyro} + 5.0032$, where $\delta^{13}C_{corr}$ is the corrected final $\delta^{13}C$ value and $\delta^{13}C_{pyro}$ is the value measured by pyrolysis and corrected with internal standards. $\delta^{13}C$ values were further corrected for the Suess effect, which is a shift in the atmospheric concentrations of carbon isotopes due to increasing fossil-fuel derived CO2 (Keeling, 1979). One-way ANOVA was performed with the SPSS® statistical package to investigate the differences between regions of the ring and between the same region of rings with and without IADFs.

3.3.7 Climate analysis

A Pearson's linear correlation function analysis ($P<0.05$) was implemented between tree-ring width, IADF frequency, $\delta^{13}C$ and $\delta^{18}O$ values of IADF wood, and monthly total precipitation and mean temperature data grouped by season (time spans: 1951-2014 for tree-ring width and IADF frequency, 1985-2006 for $\delta^{13}C$ and $\delta^{18}O$) from January of the current year to March of the next year, in order to be sure to cover all the growing season which could last up to the first months of the next year in Mediterranean species (Cherubini et al., 2003; Vieira et al., 2015). All the correlations were computed with the software R (R Core Team, 2016).

3.4 Results

3.4.1 Tree-ring width

Tree-ring width chronologies of the two species cover the same timespan from 1949 to 2014. *P. pinea* showed in the first twenty years an overall higher annual growth (6.28±0.29 mm, mean

value±SE) compared to *Q. ilex* (3.8±0.2 mm)(Fig. 2a, Table 1). After that period the annual growth of the two species is similar (1.93±0.13 mm for *P. pinea*, 2.06±0.11 mm for *Q. ilex*). The applied standardization method efficiently removed the age-effect from tree-ring width chronologies (Fig. 2b). EPS and R_{bt} values confirmed the quality of the chronologies while MS values indicated that *Q. ilex* standard chronologies were more sensitive than *P. pinea* ones (Table 1).

3.4.2 IADF frequency

In *P. pinea* and *Q.* ilex, 43% and 28% of total rings showed the occurrence of IADFs, respectively. The overall mean stabilized IADF frequency was also higher in *P. pinea* (2.54±0.22, mean value±SE) than in *Q. ilex* (1.68±0.21)($P<0.05$). Mean stabilized IADF frequency showed a good peak correspondence during time between the two species, with IADF frequency in *Q. ilex* decreasing during the period 1982 - 1991 and during the last decade, except from the year 2005 where a high frequency was found. No decrease in IADF frequency was observed for *P. pinea* (Fig. 3a). The age-effect was removed and the variations around the mean highlighted with the applied standardization method (Fig. 3b).

3.4.3 Anatomical characterization of IADFs

IADFs were positioned at the end of the ring in both species, but their morphological appearance was different. More specifically, in *Q. ilex*, tree rings without IADFs (Fig. 4a) were characterized by diffuse porosity with vessel size decreasing towards latewood, while tree rings with IADFs (Fig. 4b) showed an extra-growth band of wood located within the 75-100% of the ring width, thus successive to latewood. Such a IADF appeared as a band of dense wood where fibres and parenchima predominate and are arranged in tangential alternating bands. In *P. pinea*, tree rings without IADFs (Fig. 4c) were characterized by tracheids with decreasing lumen from earlywood to

latewood, while rings with IADFs (Fig. 4d) showed a band of earlywood-like cells within latewood, thus located at 80-100% of the ring width. In *Q. ilex*, tree rings with IADFs showed narrower vessels throughout ring width than tree rings without IADFs and were characterized by a steeper decrease of lumen area in the second half of the ring compared to rings without IADFs (Fig. 4e). In *P. pinea*, rings without IADFs showed narrower tracheids than rings with IADFs in the first half of ring width (earlywood); moreover, the band of tracheids with a wider lumen area within the latewood corresponded to the earlywood-like cells at the IADF level (Fig. 4f). Neither mean wood density nor mean vessel frequency were found to be significantly different between rings with and without IADFs in *Q. ilex* (Fig. 5a,b). Instead, both wood density and vessel frequency significantly increased from earlywood to latewood in both rings with and without IADFs, with a significantly higher latewood density in rings with IADFs than in rings without IADFs. Finally, the IADF region was characterized by a significantly higher wood density than latewood and lower vessel frequency than latewood but higher than earlywood in rings with IADFs (Fig. 5a,b). *P. pinea* measures of intra-annual wood density didn't show any significant differences between rings with and without IADFs (supplementary material, Fig. S3).

3.4.4 Isotopical characterization of IADFs

The overall intra-annual values of $\delta^{13}C$ were found to be lower in *P. pinea* than in *Q. ilex* wood (Fig. 6a,c) ($P<0.05$). The intra-annual $\delta^{13}C$ trend was quite variable inside the rings and this was also due to the high inter tree variability, showed in Table 2. However, we concentrated on the trend which was most commonly found in rings with IADFs in *Q. ilex* (50% of cases) and that was characterized by a transition from low values of $\delta^{13}C$ in the earlywood (mean value: -26.30‰), less negative values in the latewood (mean value: -26.07‰) and more negative values again in the IADF (mean value: -26.28‰) (Fig. 6a). The same trend of $\delta^{13}C$ was found for $\delta^{18}O$ (earlywood mean value: 24.92‰, latewood mean value: 25.17‰, IADF mean value: 24.93‰) (Fig. 6b), even if the

difference between ring regions (EW, LW, IADF) was not significant. Contrariwise, the intra-annual $\delta^{13}C$ trend which was most commonly found in rings with IADFs in *P. pinea* (56% of cases) was characterized by an isotopically heavier IADF wood and lower $\delta^{13}C$ values in the latewood (IADF mean value: -26.86‰, LW mean value: -28.08‰) (Fig. 6g). The same trend was found for $\delta^{18}O$, even if the difference among ring regions were not significant (Fig. 6d). Intra-annual $\delta^{13}C$ trends of rings without IADFs did not significantly increase from the earlywood to the latewood both for *Q. ilex* and *P. pinea* in most of the cases (55% and 67%, respectively), with values significantly higher compared to rings with IADFs for *Q. ilex* (earlywood mean value: -25.75‰, latewood mean value: -25.47‰), while not significantly different for *P. pinea* (Fig. 6a,c). On the contrary, the $\delta^{18}O$ most common trend in rings without IADFs slighthly increased from earlywood to latewood in *Q. ilex*, while slightly decreased in *P. pinea*, even if the differences among ring regions were not significant (Fig. 6b,d).

3.4.5 Climate signal

Q. ilex resulted more sensitive to climate than *P. pinea*, with radial growth positively driven by spring and summer total precipitation while negatively by summer mean temperature. *P. pinea* tree-ring width was only negatively correlated with winter total precipitation (Table 3). IADF frequency was driven by precipitation in *Q. ilex* while by temperature in *P. pinea*. More specifically, the occurrence of IADFs in *Q. ilex* was associated with high summer total precipitation ($r=0.29$, $P<0.05$), while IADF occurrence in *P. pinea* was associated with high mean temperature during autumn ($r=0.26$, $P<0.05$) (Fig. 7). Autumn was the season which mostly influenced $\delta^{13}C$ and $\delta^{18}O$ of IADF wood in both the species. $\delta^{13}C$ was positively correlated with autumn mean temperature ($r=0.9$, $P<0.05$) and $\delta^{18}O$ was negatively correlated with autumn mean precipitation ($r=-0.92$, $P<0.05$) for *Q. ilex*, while autumn temperature negatively influenced $\delta^{18}O$ of *P. pinea* IADFs

(r=0.74, P<0.05) (Fig. 8). $\delta^{18}O$ of the IADFs of *P. pinea* was also positively influenced by spring precipitation (r=0.69, P<0.05) (Fig. 8).

3.4.6 Soil samples

The soil was characterized by surface outcrops of volcanic rocks. The mean values of water content, available water capacity and water holding capacity in the top 10 cm of the site soil were 8.45%, 25.06% and 19.74%, respectively.

3.5 Discussion

This study allowed to identify and characterize the IADFs occurring in *Q. ilex* and *P. pinea* trees growing in the same Mediterranean stand. Information on the relationship between IADF frequency and isotopic composition in tree rings and seasonal mean temperature and total precipitation was also acquired. The significantly higher frequency of IADFs which we found in *P. pinea* compared to the co-occurring *Q. ilex* is in agreement with several studies which show a high frequency of IADFs in Mediterranean conifers and link this recurrent plastic bimodal pattern of intra-annual growth with their high sensitivity to climate fluctuations (Campelo et al., 2007b, 2013, 2015; de Luis et al., 2007, 2011; Carvalho et al., 2015; De Micco et al., 2016a; Nabais et al., 2014; Novak et al., 2013b; Olivar et al., 2015; Rozas et al., 2011; Vieira et al., 2010, 2015; Zalloni et al., 2016). The finding of *Q. ilex* IADFs as "extra-growth bands" located at the end of the ring width is in agreement with the identification given by Campelo et al. (2007a). In the same way, the identification of all the *P. pinea* IADFs of our samples within the latewood follows the tendency of Mediterranean pines to form the most of the IADFs close to the end of the ring, suggesting that IADFs are formed towards the end of the growing season (De Micco et al., 2016a). Tracheid lumen area showed to be a valuable parameter to identify and quantify IADFs in *P. pinea* rings, as an

omoxyl-wood conifer, while in the diffuse to semi-ring-porous *Q. ilex* wood, vessel frequency and wood density proved to be better indicators if compared to vessel lumen area. Wood carbon and oxygen stable isotopes analyzed together helped to understand whether changes observed in the values depend on photosynthetic capacity or stomatal conductance, since the oxygen isotope ratio composition is not determined by photosynthesis but by soil water, leaf water enrichment due to transpiration and biochemical fractionation during incorporation (Barbour, 2007; Dawson et al., 2002; Scheidegger et al., 2000). The overall higher $\delta^{13}C$ values of *Q. ilex* rings with equal $\delta^{18}O$ values compared to *P. pinea* rings, which are also shown in $\delta^{13}C$ and $\delta^{18}O$ annual curves (supplementary material, Fig. S4), indicate that a higher photosynthetic rate occurred both in years with and without IADFs of *Q. ilex* compared to *P. pinea*, since a high $\delta^{13}C$ can be the result of an increased photosynthetic capacity when not accompanied by a concomitant high $\delta^{18}O$ (Scheidegger et al., 2000). The higher photosynthetic rate of *Q. ilex* compared to *P. pinea*, which translates into a higher water use efficiency, reflects its ability to withstand drought, since it helps in the maintenance of a positive carbon balance under dry conditions (Raven, 2002). The high inter and intra-annual variability observed within the samples of the five cores left separate for the years 1985, 1995 and 2005 (Table 2), helped to explain the lack of statistical significance observed in the majority of the isotopic intra-annual values between regions of the ring due to sample pooling. However, the $\delta^{13}C$ and $\delta^{18}O$ intra-annual trends that we found in *Q. ilex* and *P. pinea* tree rings, and in particular the isotopic composition recorded in the IADF region, suggested opposite physiological behavior. *Q. ilex* showed a decrease in $\delta^{13}C$ and $\delta^{18}O$ in the IADF compared to the previous latewood, suggesting an increase of carbon supply through stomata, which means that the plant positively reacted to some climatic factor by increasing stomatal conductance (Farquhar et al., 1982; Scheidegger et al., 2000). On the contrary, *P. pinea* showed an IADF region enriched in ^{13}C and in ^{18}O compared to the other regions of the ring, which suggests a conservative response of the plant to some climatic constraint, with the decrease of stomatal conductance hence of intercellular CO_2 concentration, contributing to the increase in $\delta^{13}C$ in IADF tracheids (De Micco et al., 2007;

Farquhar et al., 1982; Scheidegger et al., 2000). The intra-annual $\delta^{13}C$ pattern that we observed in *P. pinea* often occurs in conifers, with low values in earlywood, maximum values in mid-season and declining values in the latewood (Schulze et al., 2004). The increased stomatal conductance that we related to $\delta^{13}C$ and $\delta^{18}O$ values of the IADF wood of *Q. ilex*, could find a compensation in the observed quantitative wood anatomy with the formation of a conservative hydraulic structure showing a dense wood with less vessels, which would promote the safety of water transport in order to be less vulnerable to embolism phenomena facilitated by the increased transpiration (Battipaglia et al., 2016; Carlquist, 1975, 1989; De Micco et al., 2008). On the contrary, the high values of $\delta^{13}C$ and $\delta^{18}O$, but also the larger tracheids that we found in the IADF region of *P. pinea*, could suggest an enhancement of conductive efficiency leading to a strict stomatal control with the aim to avoid dehydration (Battipaglia et al., 2013). The observed smaller lumen area of earlywood conductive elements of *Q. ilex* rings with- compared to rings without IADFs contrasts with the larger ones found in *P. pinea* rings with IADFs: this difference could reflect the intrinsic group-related differences in wood structure. The achievement of mechanical safety is more important for the eteroxyl and more efficient wood of angiosperms than for the omoxyl and safer wood of conifers (Sperry et al., 2008). We hypothesize that *Q. ilex* rings with IADFs are related to years where the trees show a safer wood structure since the beginning of the growing season, which prevents phenomena of embolism and allows the production of the "extra-growth band" of wood at the end of it wether there are favorable conditions. While *P. pinea* rings with IADFs is related to years where the trees is more sensitive to climate conditions of the early growing season achieving a more efficient hydraulic conductivity, which would make them prone to the formation of IADFs when growth conditions become harsher. For instance, Carvalho et al. (2015) suggested that IADF formation within the latewood of *P. pinaster* is predisposed by previous earlywood cell structure, in particular by higher rates of cell production in spring which, in turn, increases the number of cells under enlargement after the summer drought.

Q. ilex showed to be more sensitive to climate than *P. pinea* in terms of tree-ring growth, recording significant positive correlations with spring and summer precipitation, which concur with the water demand of *Q. ilex* trees at the beginning of the growing season and in the driest months typical of Mediterranean areas (Campelo et al., 2009; Gea-Izquierdo et al., 2009, 2011), and a negative influence of summer temperature, pointing out the decline of wood growth with drought (Di Filippo et al., 2010; Linares et al., 2009; Peñuelas et al., 2008; Piovesan et al., 2008; Sarris et al., 2011). Instead, *P. pinea* tree-ring growth seemed to be only negatively influenced by winter precipitation, which correlation does not find a straightforward explanation (perhaps, because anoxic conditions for the shallower root system of *P. pinea* developed with the amount of winter precipitation). The isotope signature of the IADFs of the two species found consistency with climate correlations with both IADF frequency and the isotopic ratio values themselves. Summer precipitation was the factor which positively influenced IADF occurrence in *Q. ilex*, while autumn temperature positively affected IADF frequency in *P. pinea* tree rings. The observed increase and decrease in IADF frequency in the last decade in *P. pinea* and *Q. ilex* rings respectively, could then be linked to the recorded rise of mean annual temperature not accompanied by an increase in precipitation during the last decades (supplementary material, Fig. S5), suggesting that the expected temperature rise and fluctuation in precipitation pattern in the Mediterranean area would affect cambial growth of these two species leading to the formation of more IADFs in *P. pinea* and less IADFs in *Q. ilex*. This would lead indeed to a higher wood productivity in *P. pinea* than in *Q. ilex*, where drought periods may trigger the reduction of transport and allocation of photoassimilates to stem and roots, preferring other resource uses (Sevanto et al., 2014). Notwithstanding, further case studies are needed to assess this preliminary forecast, and other physiological measurements have to be examined to explore the link between IADF formation and the effective adaptive capability of the species. The link between high IADF frequency and high precipitation during summer seems to be in agreement with the physiological positive response we discovered in the IADF wood of *Q. ilex* rings: favorable wet conditions of growth would lead to a less tight stomatal control with an

increase of the ratio of intercellular CO_2 to atmospheric CO_2, producing low values of $\delta^{13}C$ (Leavitt, 1993). Precipitation during summer could be related to the formation of new leaves in *Q. ilex* with the deposition of an extra growth band of xylem (Corcuera et al., 2004; Campelo et al., 2007a, 2010). Low temperature and high precipitation of autumn drove respectively the $\delta^{13}C$ and $\delta^{18}O$ composition that we found in the IADF region of *Q. ilex*, suggesting that IADFs were formed during these months. These findings confirm what Campelo et al. (2007a) suggested on the basis of climate correlations with IADF frequency: IADFs in *Q. ilex* could be triggered by summer rainfall and formed during the second growth period of the season after the summer drought, with autumn favorable conditions of growth. On the contrary, high air temperature stimulates evapotranspiration and water loss (Battipaglia et al., 2009), thus leading to the drought-avoidant response that we found in the wood of *P. pinea* IADFs with reduced stomatal conductance and high values of $\delta^{13}C$ and $\delta^{18}O$: at high temperatures photorespiration is stimulated while photosynthesis is inhibited (Rennenberg et al., 2006). Autumn seemed to be the period of IADF formation in *P. pinea* also, showing a significant correlation between IADF $\delta^{18}O$ values and mean temperature. IADFs in *P. pinea* tree rings were suggested to be formed during autumn months also by Campelo et al. (2007b), Nabais et al. (2014) and Zalloni et al. (2016), although we didn't find any significant correlation with precipitation as they noticed. Indeed, the climatic factors that control the formation of IADFs in Mediterranean pines are showed to be site-dependent and not so much dependent on the species (Nabais et al., 2014). Finally, the observed positive correlation between spring precipitation and $\delta^{18}O$ of the IADF wood of *P. pinea* could be due to the temporal shift between latewood formation and the use of water pools stored during spring (Castagneri et al., in press): the increase of $\delta^{18}O$ with increasing precipitation could be related to the shallow root system of *P. pinea* which takes water from upper soil layers enriched in ^{18}O, since evaporative isotopic franctionation decreases with soil depth (Dawson et al., 2002).

In conclusion, our study allowed the anatomical and isotopical characterization of IADFs in *Q. ilex* and *P. pinea* coetaneous trees growing in the same Mediterranean stand, which would be useful for

their accurate identification in future studies, and suggested that IADF period of formation is during autumn months for both the species. However, the influence of climate on IADF occurrence was found to be species-specific, reflecting the more anisohydric/isohydric physiological reactions to the varying environmental conditions of *Q. ilex* and *P. pinea*, respectively: an increased stomatal conductance associated to a safer wood of IADFs in *Q. ilex* while a tighter stomatal control associated to a more efficient wood of IADFs in *P. pinea*. Its assessment helped to attempt predictions about species responses to future climate: in a climate scenario of a mean temperature rise not accompanied by a total precipitation increase, *Q. ilex* would form less IADFs while *P. pinea* more. Other case studies are necessary for these species to assess the suggested forecasts, and further research is needed to link the plasticity of the species to form IADFs with their effective adaptive ability, thus with their capability to compete for resources and how it influences future population development.

3.6 References

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3.7 Figures

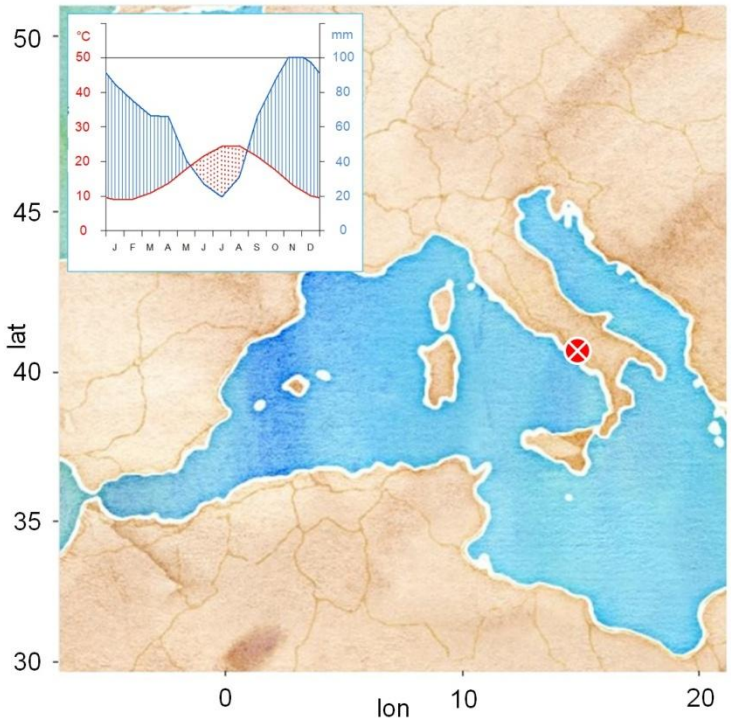

Fig. 1 Study site location and climatic diagram from the CRU TS3.23 gridded dataset at 0.5° resolution data of the period 1951-2014. (Kahle and Wickham, 2013)

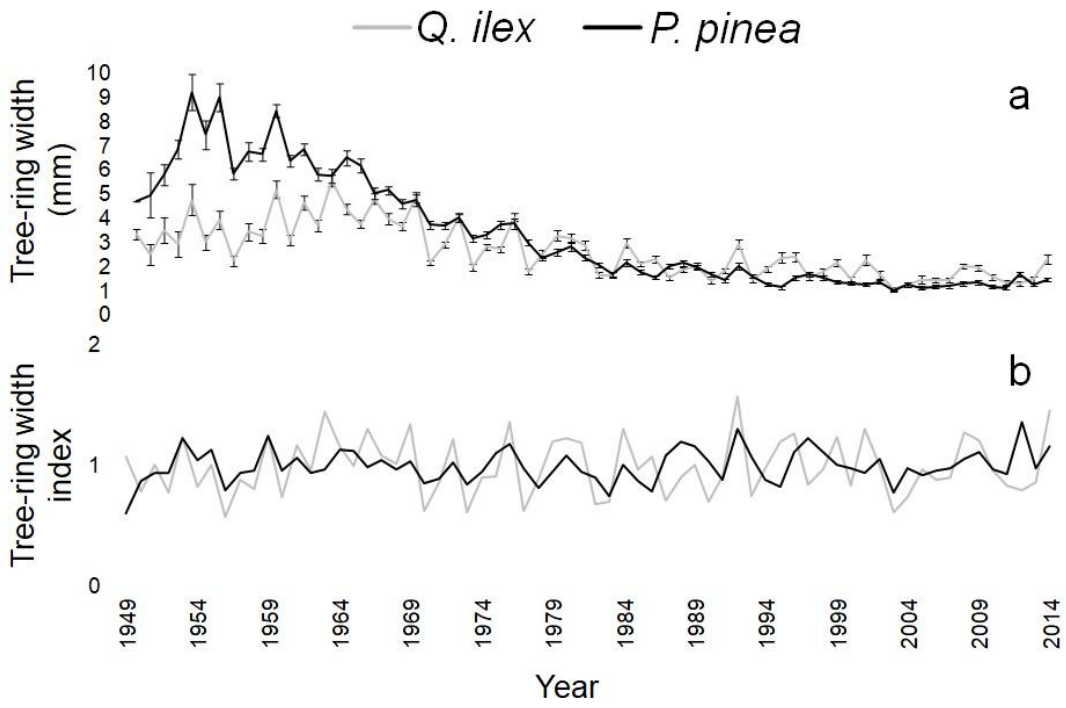

Fig. 2 Raw (a) and detrended (b) tree-ring width annual chronologies of the two species (*Q. ilex* in grey, *P. pinea* in black). Standard errors are reported for the raw chronologies.

Table 1 Descriptive features and quality assessment indexes of tree-ring width chronologies.

	Q. ilex	***P. pinea***
Timespan	1949-2014	1949-2014
Length	65	65
N cores	40	40
N trees	20	20
Mean tree-ring width ± SE (mm)	2.62±0.14	3.31±0.28
EPS	0.98	0.99
R_{bt}	0.63	0.85
MS	0.37	0.2

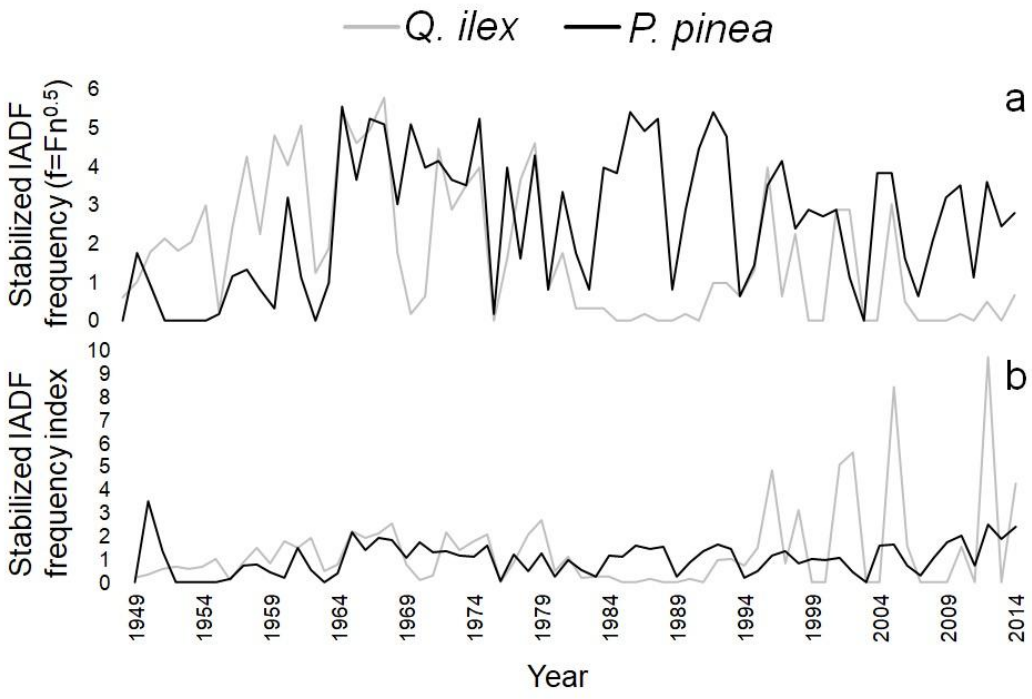

Fig. 3 Raw (a) and detrended (b) stabilized IADF frequency chronologies of the two species (*Q. ilex* in grey, *P. pinea* in black).

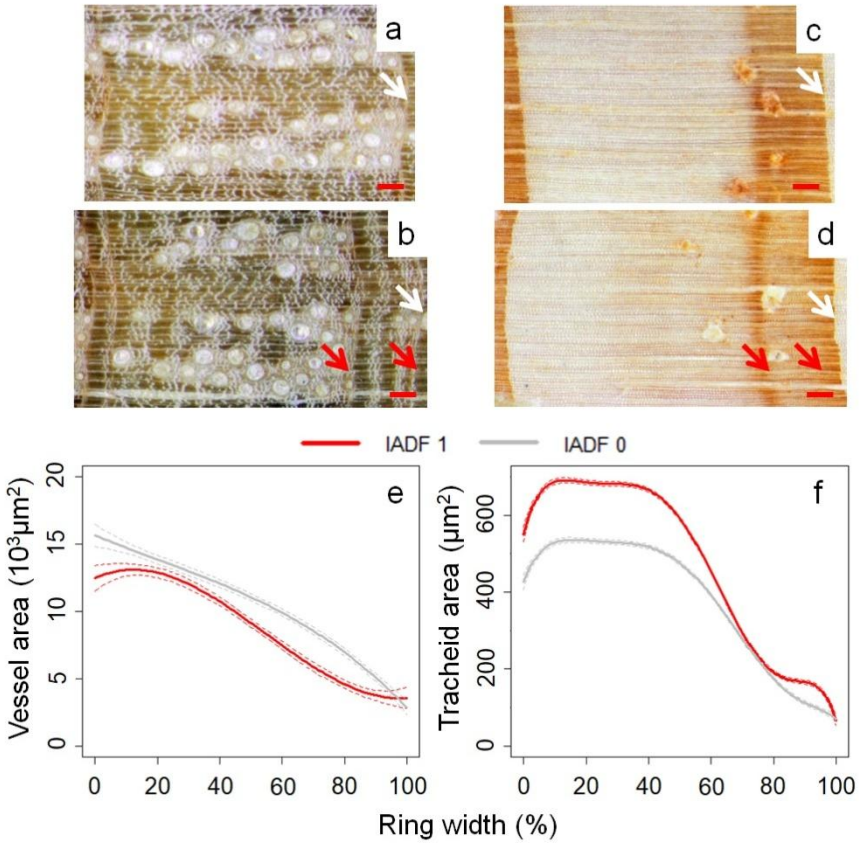

Fig. 4 Microphotographs of *Q. ilex* (a, b) and *P. pinea* (c, d) tree rings without (a, c) and with (b, d) IADFs. White arrows point the boundary of tree ring; red arrows delimit the IADF. Bars: 200 µm. Polynomial curves fitted to *in continuum* vessel area data of rings with (red) and without IADFs (grey) for *Q. ilex* (e) and *P. pinea* (f). Confidence intervals ($P<0.05$) are represented with the dotted lines.

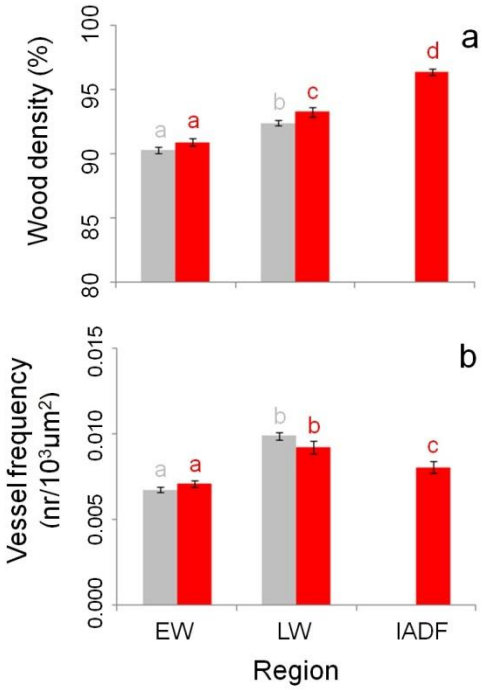

Fig. 5 Vessel frequency and wood density mean intra-annual values of intra-annual regions of *Q. ilex* rings with (in red) and without IADFs (in grey). EW=EarlyWood, LW=LateWood, IADF=Intra-Annual Density Fluctuation. Standard errors are reported. Different letters correspond to significantly different values between regions and ring type ($P<0.05$).

Table 2 $\delta^{13}C$ and $\delta^{18}O$ mean values ± standard error of each region of the ring calculated between five trees per species, for the years 1985, 1995 and 2005.

Year	Region	Species	$\delta^{13}C$ (‰ ± SE)	$\delta^{18}O$ (‰ ± SE)
1985	EW	*Q. ilex*	-25.45±0.21	23.99±0.22
1985	LW	*Q. ilex*	-25.17±0.19	24.57±0.25
1985	EW	*P. pinea*	-27.71±0.14	24.15±0.08
1985	ELW	*P. pinea*	-26.80±0.42	24.31±0.08
1985	IADF	*P. pinea*	-26.04±0.45	23.99±0.12
1985	LW	*P. pinea*	-27.62±0.02	24.5±0.07
1995	EW	*Q. ilex*	-25.84±0.22	25.14±0.21
1995	LW	*Q. ilex*	-26.24±0.07	25.36±0.15
1995	IADF	*Q. ilex*	-26.9±0.07	25.34±0.07
1995	EW	*P. pinea*	-27.48±0.39	25.06±0.15
1995	LW	*P. pinea*	-27±0.44	25.98±0.18
2005	EW	*Q. ilex*	-26.33±0.16	24.99±0.35
2005	LW	*Q. ilex*	-26.21±0.2	25.02±0.28
2005	IADF	*Q. ilex*	-26.51±0.07	24.65±0.1
2005	EW	*P. pinea*	-27.86±0.12	24.99±0.2
2005	ELW	*P. pinea*	-27.77±0.44	25.02±0.11
2005	IADF	*P. pinea*	-27.54±0.38	24.57±0.15
2005	LW	*P. pinea*	-28.74±0.31	25.21±0.31

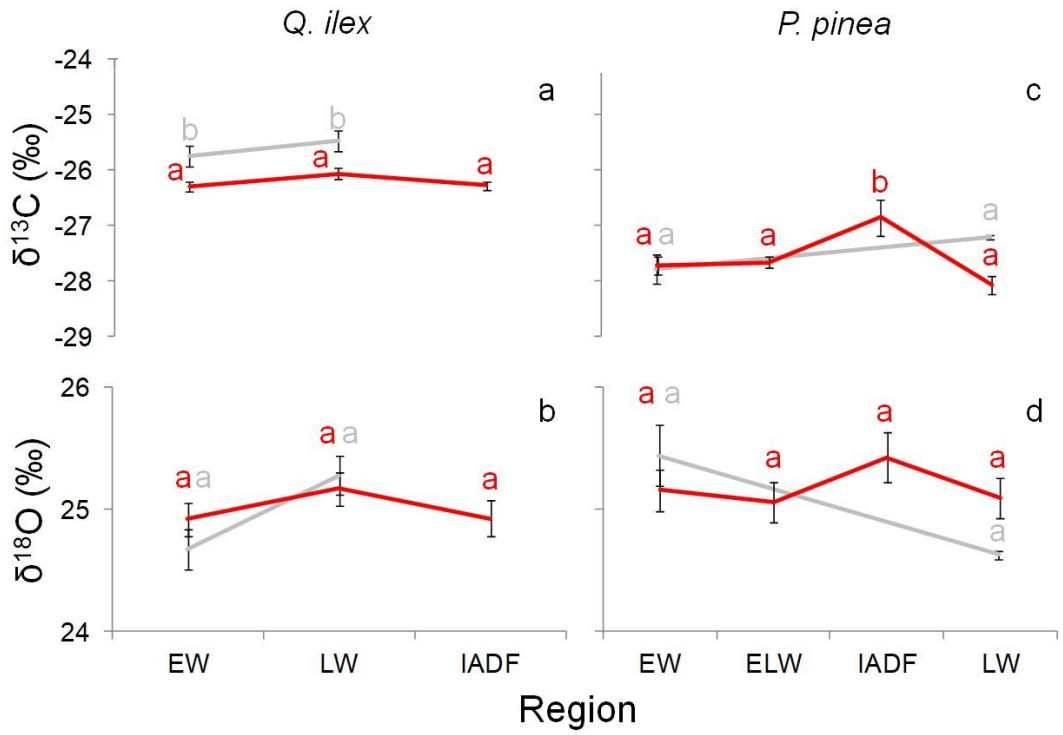

Fig. 6 $\delta^{13}C$ and $\delta^{18}O$ most common intra-annual trends of rings with (in red) and without IADFs (in grey) for *Q. ilex* (a,b) and *P. pinea* (c,d). Standard errors are reported. Different letters correspond to significantly different values between regions and ring type ($P<0.05$).

Table 3 Significant Pearson's correlation coefficients of the correlation between tree-ring width and seasonally grouped monthly mean temperature and total precipitation for the two species. WIN= from January to March, SPR= from April to June, SUM= from July to September, T= mean Temperature, P= total Precipitation. T correlations in red, P correlations in blue. ($P<0.05$)

Species	Climate parameter	Month	r
Q. ilex	T	SUM	-0.32
Q. ilex	P	SUM	0.28
Q. ilex	P	SPR	0.37
P. pinea	P	WIN	-0.29

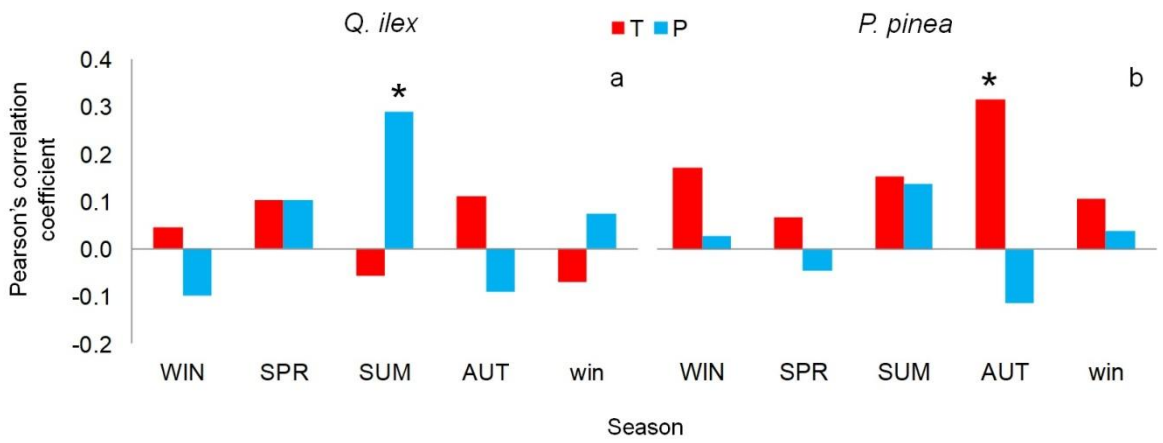

Fig. 7 Pearson's correlation between stabilized IADF frequency and seasonally grouped monthly mean temperature (T, in red) and total precipitation (P, in blue) for *Q. ilex* (a) and *P. pinea* (b). WIN= from January to March, SPR= from April to June, SUM= from July to September, AUT= from October to December, win= from January to March of the next year. ($P<0.05$)

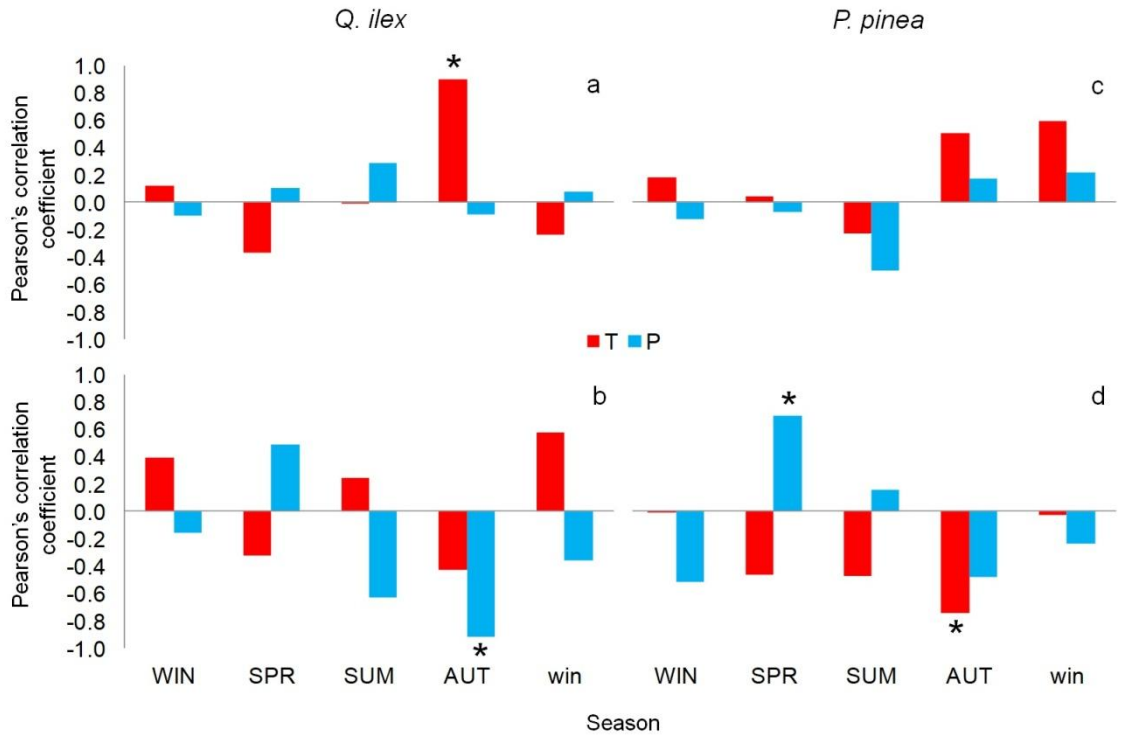

Fig. 8 Pearson's correlation between $\delta^{13}C$ (a,c) and $\delta^{18}O$ (b,d) values of IADF wood relative to the most common intra-annual trends, and seasonally grouped monthly mean temperature (T, in red) and total precipitation (P, in blue) for *Q. ilex* (a,b) and *P. pinea* (c,d). WIN= from January to March, SPR= from April to June, SUM= from July to September, AUT= from October to December, win= from January to March of the next year. ($P<0.05$)

3.8 Supplementary material

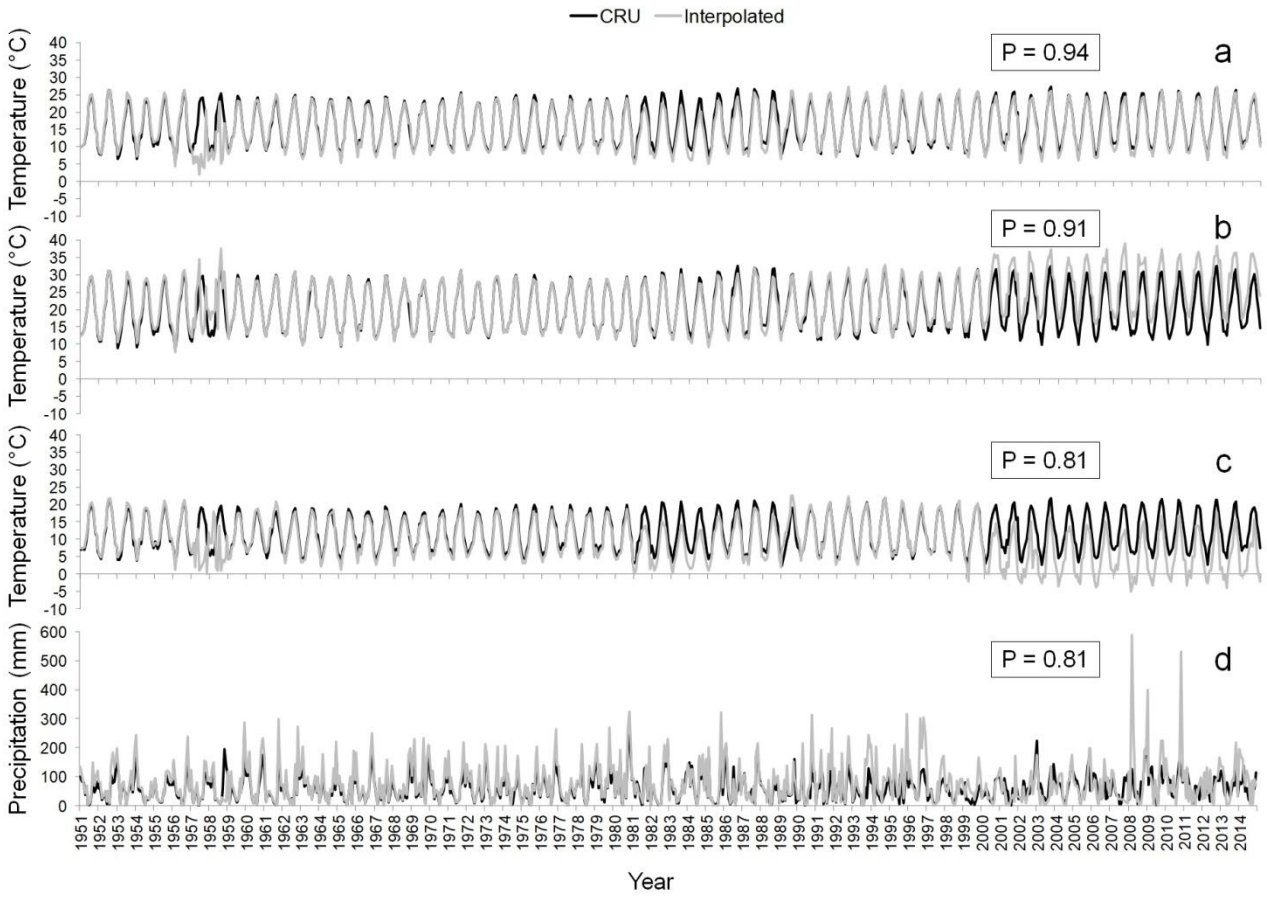

Fig. S1 Mean (a), maximum (b) and minimum (c) monthly temperature, and total monthly precipitation (d) of the CRU TS3.23 gridded dataset at 0.5° resolution data (in black)(Harris et al., 2014) and of the nearest meteorological station interpolated series (in grey) for the period 1951-2014. The values of the pearson product-moment correlation coefficient P between the two series is reported.

Table S1 Coordinates of the nearest meteorological stations from where the related climate series were interpolated.

Station	Latitude	Longitude
Osservatorio Vesuviano	40.49 N	14.24 E
Napoli/Capodichino	40.85 N	14.3 E
Ottaviano	40.85 N	14.48 E
Ercolano	40.82 N	14.37 E
Caserta	41.07 N	14.33 E
Grazzanise	41.05 N	14.06 E

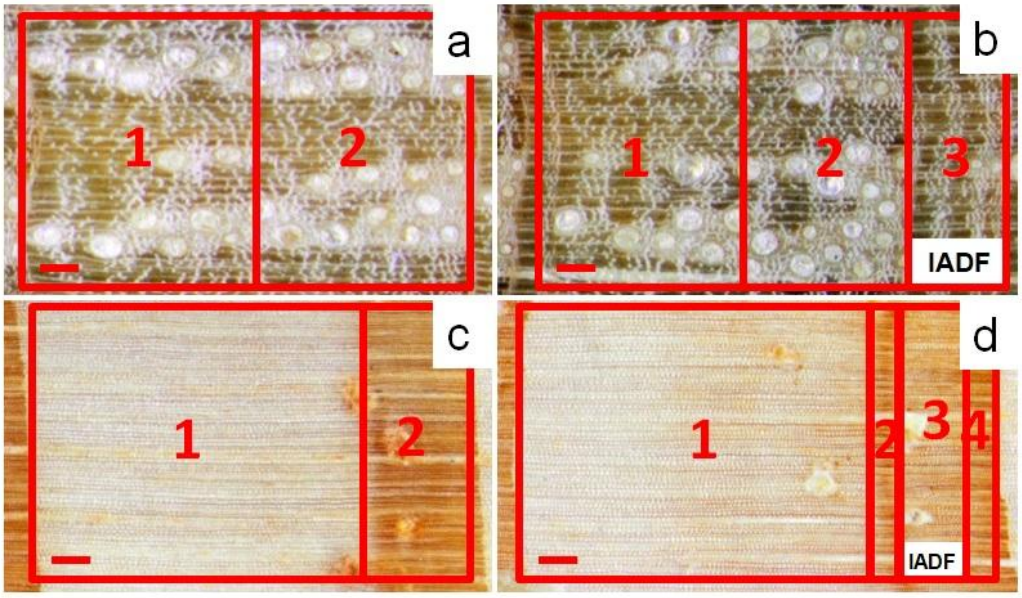

Fig. S2 Splitting of rings with (b, d) and without IADFs (a, c) in *Q. ilex* (a, b) and *P. pinea* (c, d) for stable isotope analysis. Bars: 200 µm.

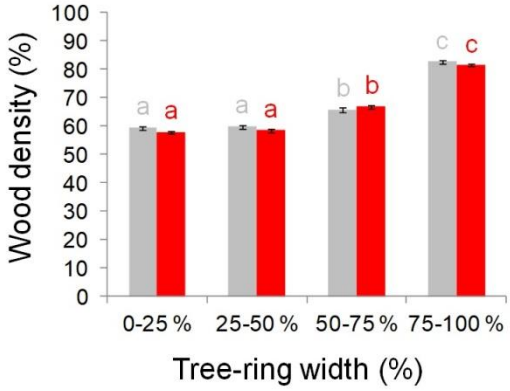

Fig. S3 Wood density mean intra-annual values of *P. pinea* rings with (in red) and without IADFs (in grey). Standard errors are reported. Different letters correspond to significantly different values between regions and ring type ($P<0.05$).

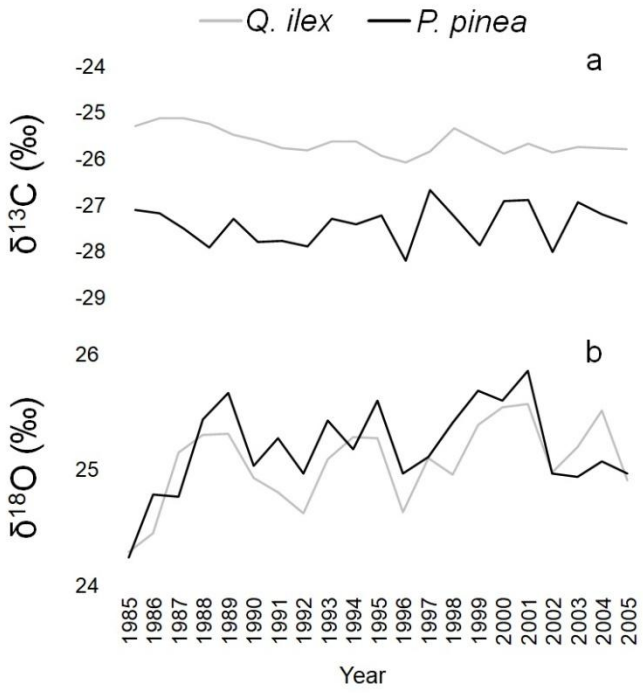

Fig. S4 Mean $\delta^{13}C$ (a) and $\delta^{18}O$ annual values (b) of *Q. ilex* (in grey) and *P. pinea* (in black) for the period 1985-2005.

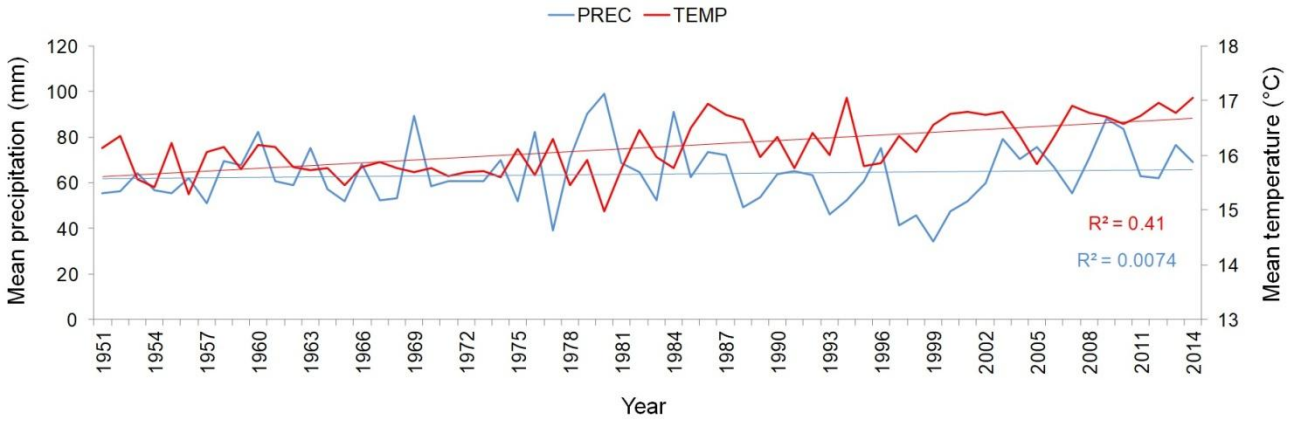

Fig. S5 Mean annual precipitation (in blue) and temperature (in red) of the CRU TS3.23 gridded dataset at 0.5° resolution data (Harris et al., 2014) for the period 1951-2014. Linear regression coefficients are reported.

4. Intra-Annual-Density-Fluctuations in tree rings of *Q. ilex* L. as functional indicators of site-specific responses to climate

4.1 Abstract

Context: Wood functional traits such as "Intra-Annual-Density-Fluctuations" (IADFs) result from the interaction between tree physiological behavior and seasonal climate changes. They could be used to reconstruct tree response to intra-annual variations in environmental conditions.

Aim: We investigated the relationship between IADF features and climate in *Quercus ilex* L. populations living on two opposite slopes, in order to search for the influence of local site conditions on wood plasticity in response to seasonal climate fluctuations.

Methods: Dominant trees were sampled and dated. IADF frequency, width and vessel size, wood density and vessel frequency were measured. Time-series climate analyses were performed with precipitation and temperature data.

Results: A decrease in IADF frequency over time negatively influenced by summer and autumn temperature was shown at both sites. IADFs were more frequent, wider, with smaller vessels and a higher wood density in trees growing at the north-slope compared to the south-slope site. IADF anatomical parameters of south-slope tree rings were influenced by precipitation, while the ones of north-slope tree rings showed to be more affected by temperature.

Conclusion: The used approach allowed to identify the main drivers for IADF formation in *Q. ilex* tree rings and to propose their ecological meaning under different environmental conditions.

4.2 Introduction

Projections of climate changes for the Mediterranean region forecast high irregularities in seasonal precipitation patterns leading to an increase in the frequency and duration of intra-annual drought events as well as a shift in time of their occurrence, accompanied by an overall increasing mean annual temperature (IPCC, 2017). These variations in climatic conditions will likely induce plastic adaptive response in trees, thus affecting plant growth and forest productivity (Lindner et al., 2010; Lindner and Calama, 2013). The study of species adaptive traits to past climatic conditions may provide information about their capacity to adapt and their vulnerability to future predicted climate changes, thus being useful in forest management. Tree responses to the changing environmental conditions have often been investigated with dendrochronology at an annual resolution to reconstruct past plant behavior (Fritts, 2001). Insights about vulnerability to peculiar intra-annual stresses, such as summer drought in Mediterranean environments, could be extrapolated from the analysis of the link between climatic parameters and wood intra-annual variability of functional anatomical traits, such as intra-annual density fluctuations (IADFs) (De Micco et al., 2016a). IADFs are defined as regions within a tree ring where abrupt changes in density occur (De Micco et al., 2016a). In Mediterranean species, IADF frequency, although related to tree age, size or tree-ring width, show severe dependency on drought: it is the result of wood functional trait adjustments induced by Mediterranean "double stress" due to summer drought and winter cold, in the pursuit of the preservation of hydraulic conductivity while reducing embolism risk (Cherubini et al., 2003, De Micco et al., 2016a). Most of the studies about IADFs within the Mediterranean region have been conducted on conifer species, showing that tree plasticity can result in high attitude to form IADFs as a reaction to climate fluctuations (Campelo et al., 2015; Carvalho et al., 2015; de Luis et al., 2011; De Micco et al., 2007; DeSoto et al., 2011; Nabais et al., 2014; Novak et al., 2013a,b; Olivar et al., 2015; Rozas et al., 2011; Vieira et al., 2010, 2015; Zalloni et al., 2016). IADF frequency has been increasing from the early 1990s to the present in *Pinus halepensis* Mill. and *Pinus pinaster* Aiton in Spain (Bogino and Bravo, 2009; Olivar et al., 2012), and seems to be higher in younger

than older trees, and in wider than narrower rings in *Pinus* spp. (Bogino and Bravo, 2009; Bräuning, 1999; Campelo et al., 2013; Copenheaver et al., 2006; Novak et al., 2013b; Olivar et al., 2012; Rigling et al., 2001, 2002; Wimmer et al., 2000; Vieira et al., 2009; Zalloni et al., 2016). Furthermore, in *P. halepensis*, *P. pinaster*, *Pinus pinea* L. and *Juniperus thurifera* L. growing around the Mediterranean basin, the presence of IADFs has been mostly related to autumn and early-summer precipitation, depending on the type and position within ring width (i.e. earlywood-like cells in latewood or latewood-like cells in earlywood) in *P. halepensis*, *P. pinaster*, *P. pinea* and *Juniperus thurifera* L. around the Mediterranean basin (Camarero et al., 2010; Campelo et al., 2007b, 2013; de Luis et al., 2007; Vieira et al., 2009; Zalloni et al., 2016). Contrariwise, less is known about IADFs in angiosperms (Battipaglia et al., 2010, 2014; Campelo et al., 2007a; Cherubini et al., 2003; Copenheaver C.A., 2010; De Micco and Aronne, 2009; Zhang and Romane, 1991). Among Mediterranean hardwoods, *Quercus ilex* L. trees growing in Spain and France were shown to form IADFs with a higher frequency in wider than in narrower rings; such IADFs were considered as a wood growth reaction to the occurrence of precipitation after summer drought periods (Campelo et al., 2007a; Zhang and Romane, 1991). A relationship has been also reported between IADF width of *Q. ilex* and the amount of precipitation during the second part of the growing season, suggesting the potentiality of using IADFs in this species as indicators of xylem phenotypic plasticity (Campelo et al., 2007a). *Q. ilex* is a widespread Mediterranean diffuse-porous hardwood species, native to southern Europe. It is widely distributed throughout the Mediterranean Basin as a dominant species in a transition zone between temperate forests and shrublands, where a very sensitive response to climate is expected, since trees growing at the edge of their distribution react more distinctly to limiting environmental factors (e.g. González-González et al., 2014; Gruber et al., 2010). Moreover it is representative of a key Mediterranean vegetation functional type, being an evergreen sclerophyll drought-resistant species (Terradas, 1999). The study of the IADF occurrence in *Q. ilex* through the investigation of the link between climate and wood anatomical traits with intra-annual resolution improves the information about the climatic signals held in tree

rings if compared to the understanding achievable from the analysis of the sole tree-ring width (Campelo et al., 2010). Such a deeper anatomical analysis of IADfs may contribute to shed light on the relationship between wood structure and function and adds insights into the ability of a widespread Mediterranean species to cope with the foreseen climate changes. Taking different stand structures and microclimates into account might help to add detail to the study: i.e. opposite slopes, even in the short distance, may differ in light intensity, soil and air temperature, humidity, soil moisture and evaporation, duration of growing periods and especially solar radiation received, thus influencing plant growth (Bennie et al., 2006; Cantlon, 1953; Kirchhefer, 2000; Kutiel and Lavee, 1999; Leonelli et al., 2009; Oberhuber, 2004; Pook and Moore, 1966; Sternberg and Shoshany, 2001). Within the northern hemisphere, south-facing slopes are generally warmer and less humid, receive more solar radiation, and are characterized by drought-resistant vegetation if compared to North-facing exposures (Måren et al., 2015). In this paper, we investigated: 1) the growth response of *Q. ilex* populations living on two opposites slopes by analyzing the climate signal in tree-ring width and wood anatomical traits in long tree-ring chronologies, 2) the relationship between IADF occurrence, IADF width and IADF wood anatomical traits and temperature and precipitation. Our working hypotheses were the following: i) tree- growth is positively influenced by the two main Mediterranean precipitation periods in spring and in autumn, while negatively by summer dry period, with mean annual vessel area, wood density and vessel frequency chronologies showing higher correlations with climate than tree-ring width; ii) IADF frequency is higher at the southern-slope site, where the higher exposure to solar radiation should increase drought intensity and frequency during summer; iii) IADFs have a wider width at the northern-slope site because of higher humidity which would help to lengthen the benefit of precipitation during autumn; iv) anatomical traits of the IADFs are related to temperature and precipitation of the warmest months of the season, and the parameters are influenced mostly by different climate factors depending on the site.

4.3 Materials and Methods

4.3.1 Study sites

The study sites are located on two opposite slopes of the Somma-Vesuvio volcanic complex, at about 15 km SE of Naples (Southern Italy). The two sites are characterized by different aspect and vegetation: the SW-faced site (SW, lat 40.49050 N, lon 14.24124 E), located in the "Tirone Alto-Vesuvio" Forest State Reserve, is covered by Mediterranean maquis and sclerophyll forests, whereas the NE-faced site (NE, lat 40.49902 N, lon 14.27067 E), located on the northern slopes of Mount Somma, is mostly characterized by broadleaved mesophilous forests. Both sites are dominated by *Quercus ilex* L. with a *Q. ilex* understory and the sporadic presence of *Robinia pseudoacacia* L., a non-native invasive species. Mean diameter and height of sampled trees were respectively 33.72 ± 0.81 cm (mean value±SE) and 16 m at SW, while 24.36 ± 1.64 cm and 13 m at NE. Tree density was 11000 and 33000 tree/ha at SW and NE respectively.

Temperature and precipitation data from the nearest meteorological stations were fragmented and did not cover the whole study period; therefore they were interpolated and compared to the CRU TS3.23 gridded dataset at 0.5° resolution data for the period 1951-2014 (Harris et al., 2014). Since the correlation between the two data series was significant (supplementary material, Chapter 2, Fig. S1, Table S1, pag. 83), we considered the climate data from the CRU to be representative for the study sites. The climate is Mediterranean with mild winter and warm summer with a mean annual temperature of 16.1°C and precipitation mainly concentrated in autumn and winter and at the beginning of spring, with an average of 766 mm per year (reference period 1951-2014).

4.3.2 Soil samples

Water content, available water capacity and water holding capacity were determined by taking 6 samples per site and following standard procedures (USDA, Natural Resources Conservation

Service, National Soil Survey Center, 1996). Undisturbed soil was collected with a PVC 10-cm cylinder with known weight filled up to the top. Each cylinder was sealed at the lower end with gauze and brought to the lab in a plastic bag without overturn it. The samples were then weighted (fresh weight) and placed in a few inches of distilled water for 48 hours to allow the water to rise by capillarity. Then, after the loss of the excess of water by percolation, samples were weighted to determine field capacity (the optimal condition of soil water content with saturated micropores). The soil of each sample was finally moved into aluminum bowls and placed in a stove at 105°C. Dry weight was determined when the samples reached a constant weight after drying.

4.3.3 Tree-ring data

Only dominant *Q. ilex* trees were sampled (twenty-one at SW and fourteen at NE) taking two incremental cores per tree at breast height (1.3 m), with a 0.5 cm diameter increment borer (Haglöf Sweden), following standard methods (Schweingruber, 1998). The number of cored trees at each site was different because no more dominant trees were available at the NE. The increment cores were air dried, mounted on wooden supports and sanded with progressively finer grades of sandpaper (up to 600 grit) to provide a flat surface, in order to clearly identify tree-ring anatomical traits under magnification. Tree rings were visually crossdated and tree-ring width was measured with a resolution of 0.01 mm using a Leica MS5 stereoscope (Leica Microsystems, Germany) fitted with a LINTAB measuring system (Frank Rinn, Heidelberg, Germany) and analyzed with the TSAP-Win software. Cross-dating accuracy of the individual series was checked using TSAP-Win and the software COFECHA, which performs the segmented time-series correlation technique (Holmes, 1983). The mean chronologies of the sites and chronology quality assessment were implemented with the software R using the Dendrochronology Program Library (dplR; Bunn, 2008, 2010). Chronology quality was assessed calculating the total correlation between trees (R_{bt}), as a measure of common signal strength, and the expressed population signal (EPS) which defines how

well a chronology, based on a finite number of trees, approximates the theoretical true population chronology referring to the threshold value of 0.85 (Wigley et al., 1984). Age and low-frequency growth trends were removed in order to perform climate correlations by fitting a smoothing spline curve with a 50% frequency response cutoff and a rigidity of the 67% of series length to the individual series (Cook and Peters, 1981). The observed values were divided by the estimated spline curves to obtain indexes, and an autoregressive model was performed to remove the first-order autocorrelation. Mean detrended chronologies of the sites were then obtained by averaging the index series using a biweight robust mean to reduce the influence of outliers. Mean sensitivity (MS) was calculated on detrended series; it indicates the mean percentage change from each measured yearly ring value to the next (Fritts, 1976).

4.3.4 Intra-Annual Density Fluctuation data

IADF occurrence was detected within the rings of each core by observing them under a stereomicroscope. IADFs were identified by detecting variations in cell lumen area, frequency and wall density different from the "standard" transition from earlywood to latewood described in Wheeler (2011), as found in Campelo et al. (2007a) and defined in Chapter 1. Relative annual IADF frequency chronologies of the sites were calculated as the ratio between the number of cores with an IADF and the total number of cores per each year. Stabilized annual IADF frequency chronologies were then calculated according to Osborn et al. (1997) as $f = Fn^{0.5}$, where F is the relative IADF frequency value and n is the total number of cores per each year, in order to stabilize the variance and to overcome the issue of the changing sample depth over time. To investigate age- and ring-width-related growth trends, an age- and a ring-width- based IADF frequency chronology were calculated per each site following the same procedure, but by aligning the individual series of IADF presence/absence by cambial age (considering pith-offset estimations) and by classes of ring width respectively (Zalloni et al., 2016). Polynomial curves were then fitted to the data points to explore

the relationship between age/ring-width and IADF frequency observed per each age/ring-width. Age trend was then removed by dividing the best fitted estimated polynomial curves for the age-based chronologies from the original observed values, in order to perform climate correlations.

IADF width was measured with a resolution of 0.01 mm using the measuring system used for measuring ring widths. Mean IADF-width chronologies of the sites were built. IADF-width values were then converted in IADF width/tree-ring width percentage values in order to avoid tree-ring width related biases. A mean IADF-width chronology based on the new percentage values was then calculated per each site in order to perform climate correlations.

4.3.5 Quantitative wood anatomy

Tree-ring microphotographs of all the rings from five cores per site (314 rings for SW and 182 rings for the NE) were taken under a dissection microscope (SZX16, Olympus, Germany), equipped with a XM10 camera (Olympus), after filling the vessels with chalk in order to maximize the contrast and make them more visible (Gärtner & Schweingruber, 2013). Intra-annual anatomical parameters were quantified with the digital image analysis software Analysis 3.2 (Olympus). Vessel lumen area was quantified *in continuum* from the beginning to the end of each ring along a transect between two rays (with an area between 378.87 and 9944.156 $10^3 \mu m^2$ for SW and between 2724.28 and 26535.23 $10^3 \mu m^2$ for the NE) (De Micco et al., 2014). For the quantification of wood density and vessel frequency parameters, the same transects were divided into four equal regions (with each region corresponding to the 25% of total ring width). Wood density was quantified as the percentage of cell walls over total xylem area per each region (De Micco et al., 2016b), while vessel frequency was calculated as the number of vessels per $10^3 \mu m^2$, determined by counting vessels in a known area according to Wheeler et al. (1989). Tree-ring mean annual values of each anatomical parameter were calculated and mean chronologies were built. Age and low-frequency growth trends were removed in order to calculate climate correlations with the same method applied to tree-ring

width series to avoid differences associated with detrending procedures (Campelo et al., 2010). Chronology quality was assessed as for tree-ring data. Mean IADF annual values of vessel lumen area, wood density and vessel frequency were calculated and mean chronologies were implemented in order to perform climate correlations.

4.3.6 Climate signal

The climate signal of all the series was investigated with a bootstrapped correlation function analysis (Pearson's linear correlation, $p<0.05$) with seasonally grouped monthly total precipitation and mean temperature data from December of the previous year to December of the current year, for the different growth time spans covered by the trees of the two sites: a) 1952-2014 for tree-ring width, IADF frequency, IADF width and anatomical parameters series of SW, b) 1966-2014 for tree-ring width, IADF frequency, IADF width and anatomical parameters series of NE. All the correlations were computed with the software R using the treeclim package (Zang & Biondi, 2015).

4.4 Results

4.4.1 Tree-ring data and anatomical traits

Descriptive statistics and quality assessment indexes of tree-ring mean chronologies are shown in Table 2. Mean chronologies of the NE covered a shorter period compared to the SW ones: NE trees were about twenty years younger than SW trees. Tree-ring width chronologies (TRW) showed the highest EPS and MS values. EPS values of the anatomical parameters chronologies were lower than the critical value of 0.85 except for the mean vessel area chronology (MVA) at the NE, and reaching the lowest values for vessel frequency chronologies (VF). VF also showed the lowest values of R_{bt}, while wood density chronologies (WD) were very complacent with consistently low MS values.

Raw and detrended mean annual chronologies are showed in Figure 2. NE trees showed wider rings (2.47 ± 0.12 mm, mean value ± SE), with smaller vessels (24.45 ± 0.93 $10^3 \mu m^2$), a denser wood (94.63 ± 0.2 %) and similar vessel frequency (0.0022 ± 0.00005 vessels/$10^3 \mu m^2$) compared to SW trees.

4.4.2 IADF data

IADFs were more frequent and wider in SW tree-rings than NE ones, even considering the common period aligned by cambial age (Table 3). IADFs of SW tree-rings showed larger and more sparse vessels with a less dense wood than NE IADFs. High values of standard deviations for all the mean parameters indicate a high variability among years. An age-trend was found: IADFs were more frequently formed when trees were younger at both sites (Fig. 3). Trees from both sites showed the same tendency, with the occurrence of more IADFs in wide rings than in very narrow or very large rings, which is observable in the bell-shaped distributions in Figure 3. The highest IADF frequency was found between 3-4 mm for SW tree-ring widths while between 2-3 mm for NE ones, despite SW trees showed to have more narrow rings than NE ones (Fig. 3). Both sites showed an IADF frequency decrease during the most recent growth years in the stabilized IADF frequency chronologies, while NE tree rings showed the occurrence of IADFs during periods of no-occurrence for SW ones (Fig. 4). Stabilized IADF frequency chronologies of the two sites didn't show a high peak coincidence of their occurrence, which was instead found between the IADF width chronologies of the two sites (Fig. 4).

4.4.3 Climate signal

Tree-ring width was positively influenced by spring (SPR-from current March to current June) precipitation at both sites ($r=0.317$ for SW, 0.265 for the NE, $P<0.05$), while only SW tree-ring

width showed a positive correlation with autumn (AUT-from current September to current December) precipitation (r=0.230, P<0.05) (Fig. 5). SW tree-ring width showed a negative significant correlation with summer (SUM-from current June to current September) temperature (r=-0.257, P<0.05) (Fig. 5). Tree-ring mean vessel area presented no significant correlation with climate, except for SW mean vessel area which was negatively correlated with summer precipitation (r= -0.249, P<0.05) (Fig. 5). Mean annual wood density and vessel frequency did not show significant correlations (supplementary material, Fig. S2). IADF frequency and width were influenced by mean temperature rather than total precipitation: mean temperatures from spring to autumn were negatively correlated with IADF frequency (r=-0.376 (SPR), 0.315 (SUM), 0.226 (AUT), P<0.05) (Fig. 6). Summer (r=-0.384, P<0.05) and autumn (r=-0.433, P<0.05) temperatures were negatively correlated with IADF width in SW trees (Fig. 6). IADF frequency was negatively influenced by autumn temperature (-0.133, P<0.05) and IADF width by spring (-0.454, P<0.05) and autumn (r=-0.433, P<0.05) temperature in NE trees (Fig. 6). IADF frequency was also negatively influenced by spring precipitation in NE trees (r=-0.166, P<0.05) (Fig. 6). Precipitation seemed to have no effect on IADF width at both sites. Since IADFs were located within the last 25% of the total ring width, mean annual chronologies of IADF anatomical parameters were built considering the region which corresponded to the latest 25% of total ring width. IADF anatomical parameters of SW tree rings were influenced by precipitation, while the ones of NE tree rings were mainly affected by temperature (Fig. 7). Summer and autumn precipitation positively influenced IADF mean vessel area in SW tree rings (r=0.469 (SUM), r=0.379 (AUT), P<0.05), while summer precipitation negatively and positively affected IADF wood density and vessel frequency, respectively (r=-0.449, r=0.448, P<0.05). Spring and summer mean temperature were positively correlated with IADF wood density (r=0.344 (SPR), r=0.430 (SUM), P<0.05) while summer temperature was negatively correlated with IADF vessel frequency in NE tree rings (r=-0.366, P<0.05) (Fig. 7).

4.4.4 Soil samples

The soil of the two sites were different in available water capacity and water holding capacity, at a same water content: the soil at SW showed higher available water capacity (41.08%) and water holding capacity (28.79%), and a water content of 11.67% compared to the soil at NE (18.17%, 15.23% and 10.51%, respectively).

4.5 Discussion

An age-trend was found in the IADF frequency chronologies at both sites which was in agreement with other similar findings in Mediterranean hardwoods (De Micco et al., 2016a), as well as in Mediterranean pines in Italy, Portugal and Spain (Zalloni et al., 2016). Younger trees could be more prone to form IADFs than older ones because of a higher sensitivity to environmental fluctuations, due to the higher grow rate for biological trend, as well as to the tree's morphology and physiology (e.g. the shallower root system which does not allow to reach deep soil water sources) (Battipaglia et al., 2014; De Micco et al., 2016; Zalloni et al., 2016). A high frequency of IADF occurrence in wider than narrower rings in *Q. ilex* is also in agreement with findings by Campelo et al. (2007a) in northeast Spain and Zhang and Romane (1991) in Southern France. While the same bell-shaped distribution with the occurrence of more IADFs in wide rings than in very narrow or very large rings was found in pine species throughout the Mediterranean basin, relating very wide rings to years without marked fluctuations in environmental conditions and very narrow years to yearly unfavorable conditions of growth which could not allow the resumption of cambial activity linked to the formation of IADFs (Zalloni et al., 2016). Our results support our first working hypothesis: tree-ring width was mainly driven by spring precipitation at both sites, but with a stronger influence at SW; autumn was the other precipitation driving period and summer temperature also seemed to play a significant negative role. The increase in radial growth was found positively linked with spring precipitation in previous studies on *Q. ilex* (Campelo et al., 2009; Gea-Izquierdo et al., 2009,

2011), as in other Mediterranean oaks (Di Filippo et al., 2010; Romagnoli and Codipietro, 1996), indicating the importance of an adequate water availability at the beginning of the growing season, in order to cope with the water shortage of the following summer dry period. The significant negative correlation between ring width and summer temperature that we found only for one site, suggests a stronger influence of the drought period on secondary growth of SW than of NE. South-facing slopes have a longer photoperiod but evapotranspiration is also higher, increasing drought stress in summer (Gratani et al., 2008) than north-facing slopes. Site-specific tree-ring growth responses to climate drivers were recorded also for *P. pinea* trees in Italian coastal stands (Mazza et al., 2014). Tree-ring growth decline with increasing summer drought has been reported in Mediterranean oaks (Di Filippo et al., 2010; Gea-Izquierdo et al., 2009, 2011, Colangelo et al., 2017), as well as in other species (Linares et al., 2009; Peñuelas et al., 2008; Piovesan et al., 2008; Sarris et al., 2011, Fernández-de-Uña et al., 2017). At the same time, the positive relationship between tree-ring width of SW and autumn precipitation of the current year could reflect a marked autumnal period of growth, which could be linked with the formation of IADFs (Battipaglia et al., 2010; Gea-Izquierdo et al., 2011, 2013). Indeed, IADFs were more frequent, wider and with larger vessels in trees at SW compared to trees at the NE. Marked seasonal fluctuations in environmental conditions as the succession of dry summer and wet autumn, are considered to be the main requirement for IADF formation (Carvalho et al., 2015; Zalloni et al., 2016). The absence of significant relationships between radial growth and summer and autumn climate for the NE could suggest that cambial activity often ceased earlier or it was quite slow (Cherubini et al., 2003), i.e., the influence of the alternation between dry summer and wet autumn affected less NE-faced trees' growth, leading to the formation of a lower number of IADFs. In agreement with this, tree-ring width was wider at NE than at SW. Nevertheless, the IADFs at the NE site were narrower, which leads us to partly reject our third hypothesis. The soil of the NE, with lower available water capacity and water holding capacity compared to SW, could assure low water reserves after the period of summer drought if autumn precipitation does not provide soil recharge (Pumo et al., 2008), leading

to the formation of a narrower growth band. Moreover, the younger trees of the NE growing in a denser stand, could have shallow root systems and may be less able to access the deep soil layers, besides competing more for water resources of the top soil layer, thus have a lower capability to profit from the autumnal precipitation period (Battipaglia et al., 2014). Indeed, IADF frequency was negatively related to autumn temperature at the NE, suggesting that soil moisture supply could be limiting with high temperature, i.e. because of direct evaporation, and thus not allow a substantial re-growth with the formation of IADFs. Temperature was the factor limiting IADF occurrence at SW also, where high mean temperature from spring to autumn would lead to the formation of less IADF within the rings. Cambial activity may slow down or even ceases under intense drought (Babst et al., 2016; Battipaglia et al., 2010; de Luis et al., 2011; Vieira et al., 2015), preventing the formation of IADFs, as suggested in Zalloni et al. (2016). Therefore, the decrease of IADF frequency observed through time at both sites, would be linked to the rise of mean annual temperature during the last decades, suggesting that the expected temperature increase in the Mediterranean area would affect cambial growth in *Q. ilex* likely inducing an earlier stop, not allowing the autumnal secondary re-growth which would lead to the formation of IADFs. This hypothesis is consistent with the stop of primary growth in this species in response to summer water deficit (Gratani, 1996; Montserrat-Martí et al., 2009). IADF width chronologies showed the same negative correlation with temperature, but with higher correlation coefficient values than IADF frequency chronology ones. Therefore, considering also the higher peak coincidence observed between IADF width chronologies of the two sites compared to IADF frequency ones, which could be related to the strong common driving climate signal found, we suggest IADF width as a more valuable parameter to compute climate correlations than the mere IADF occurrence.

Our fourth working hypothesis can also be accepted. Anatomical traits of IADF wood resulted mainly related to summer climate, thus the hottest and driest season of the year, showing temperature and precipitation as the main triggering IADFs factors but with a different role respectively for NE and SW. Temperature and precipitation as the predominant climatic drivers of

wood anatomical traits respectively at a northern and at a southern site were found also in the Mediterranean shrub *Erica arborea* L. by Gea-Izquierdo et al. (2013). In our study, high temperatures during spring and summer would lead to the formation of a conservative wood structure with a dense IADF wood also due to lower vessel occurrence in the NE trees, which promotes the safety of water transport with the increase of wood strength, which is directly proportional to embolism resistance (Battipaglia et al., 2016; Carlquist, 1975, 1989; De Micco et al., 2008). On the contrary, summer and autumn precipitation were the driving factor of IADF anatomical traits at SW, leading to the formation of large and less sparse vessels thus to a lighter wood compared to the NW. It seems that a more efficient strategy was carried out within the IADFs of SW, maintaining a high conductivity when water was available even if with a less safe wood (Sperry et al., 2008; Tyree and Zimmermann, 2002). As for IADF frequency and width, anatomical structure of IADFs at the NE was limited by high temperature, according to the hypothesis of summer drought limiting soil moisture supply. Contrarily, water availability played the role of driving the adaptive plasticity of IADF xylem at SW, where trees seemed to be more sensitive to precipitation seasonal fluctuations but showing to be more capable to take advantage of them at the same time. The different wood anatomy structure of the IADFs found in the two sites suggests that summer climatic conditions could induce a different reaction in SW and NE trees, when temperature and precipitation conditions are favorable to growth during the following autumn. A more efficient strategy with high conductivity, wide radial growth, but high risk of embolism could be experienced in the IADFs of the SW trees, while a more conservative behavior with low conductivity, narrow radial growth and low vulnerability to embolism for the IADFs of the NE trees. Further studies are needed to assess the potential link between *Q. ilex* IADF anatomical traits and physiological responses to drought. Furthermore and finally, results partly refuted our first working hypothesis: anatomical trait mean chronologies did not provide a better climatic signal than tree-ring width, disagreeing with Campelo et al. (2010), since they showed no significant

correlation except for the one between high mean annual vessel area and drier summer in SW tree rings.

4.6 Conclusions

The analysis of the climate signal in IADF occurrence and anatomical trait chronologies in *Q. ilex* tree rings provided insights on the relationship between wood structure and growth response to temperature and precipitation at the two different sites. Radial growth was strongly influenced by the alternation between dry summer and wet autumn only at SW, leading to more frequent and wider IADFs compared to the NE. An age and a tree-ring width trend were found in IADF frequency of *Q. ilex* of both the study sites. Temperature was the factor limiting IADF occurrence at both sites, suggesting that the expected temperature increase in the Mediterranean area would affect cambial growth in *Q. ilex* likely inducing an earlier stop, not allowing the autumnal secondary re-growth which would lead to the formation of IADFs. Furthermore, we found that IADF width has a better climatic signal than IADF frequency of *Q. ilex* in our study sites. Climatic correlations with anatomical traits of the IADFs helped to distinguish between different predominant climatic drivers of the two sites. Summer and autumn precipitation lead to the formation of IADFs with an efficient wood in the SW trees, with large and less sparse vessels. While spring and summer temperature influenced the anatomical structure of IADFs of the NE trees, leading to a conservative strategy with less vessels and a denser wood. The differences we found between sites could be ascribed to differences in aspect, soil water holding capacity and stand density. Our findings which showed differences in IADF occurrence and anatomical structure between two close but different sites, suggest that further research is needed to assess how climate change impacts can vary depending on stand structure and local site conditions. Improving the knowledge about species adaptation traits could provide information about their capacity to adapt in different site conditions and their vulnerability to future predicted climate changes, thus be useful in forest management choices.

4.7 References

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4.8 Figures

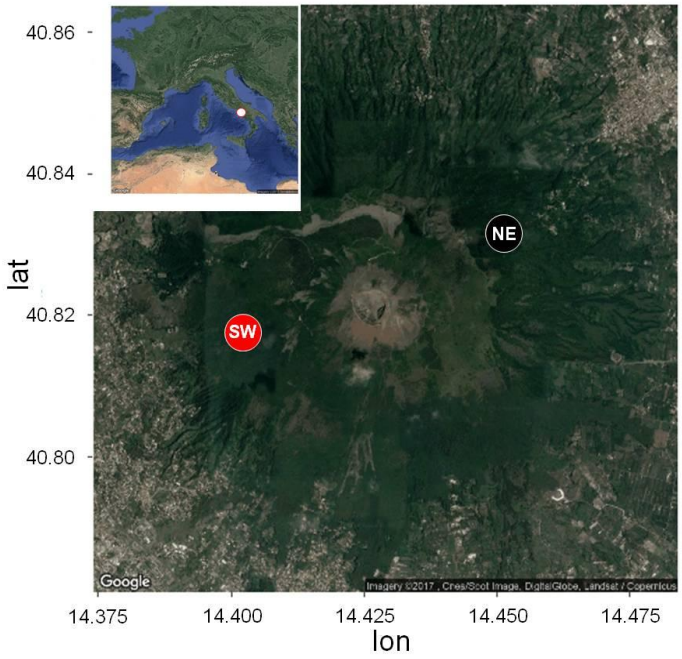

Fig. 1 Location of the two study sites (**SW**=South-West and **NE**=North-East site). (Kahle and Wickham, 2013)

Table 1 Coordinates and altitude of the two study sites (**SW**=South-West and **NE**=North-East site).

	SW	NE
Latitude (°)	40.49050 N	40.49902 N
Longitude (°)	14.24124 E	14.27067 E
Elevation (m a.s.l.)	528	669

Table 2 Descriptive features and quality assessment indexes of tree-ring mean annual chronologies (**TRW**=Tree-Ring Width; **MVA**= Mean Vessel Area; **WD**= Wood Density; **VF**= Vessel Frequency; **SW**= South-West site; **NE**= North-East site).

	TRW (mm)		**MVA (10^3µm^2)**		**WD (%)**		**VF (N vessels/10^3µm^2)**	
	SW	**NE**	**SW**	**NE**	**SW**	**NE**	**SW**	**NE**
Timespan	1948-2015	1966-2015	1950-2015	1971-2015	1950-2015	1971-2015	1950-2015	1971-2015
Length	68	50	66	45	66	45	66	45
N cores	42	28	5	5	5	5	5	5
N trees	21	14	5	5	5	5	5	5
Mean value ±SE	2.38 ±0.16	2.47 ±0.12	33.07 ±0.98	24.45 ±0.93	93.35 ±0.22	94.63 ±0.2	0.0021 ±0.00008	0.0022 ±0.00005
EPS	0.86	0.91	0.69	0.85	0.79	0.65	0.37	0.26
R_{bt}	0.81	0.4	0.31	0.58	0.44	0.31	0.11	0.05
MS	0.35	0.33	0.21	0.17	0.02	0.01	0.24	0.22

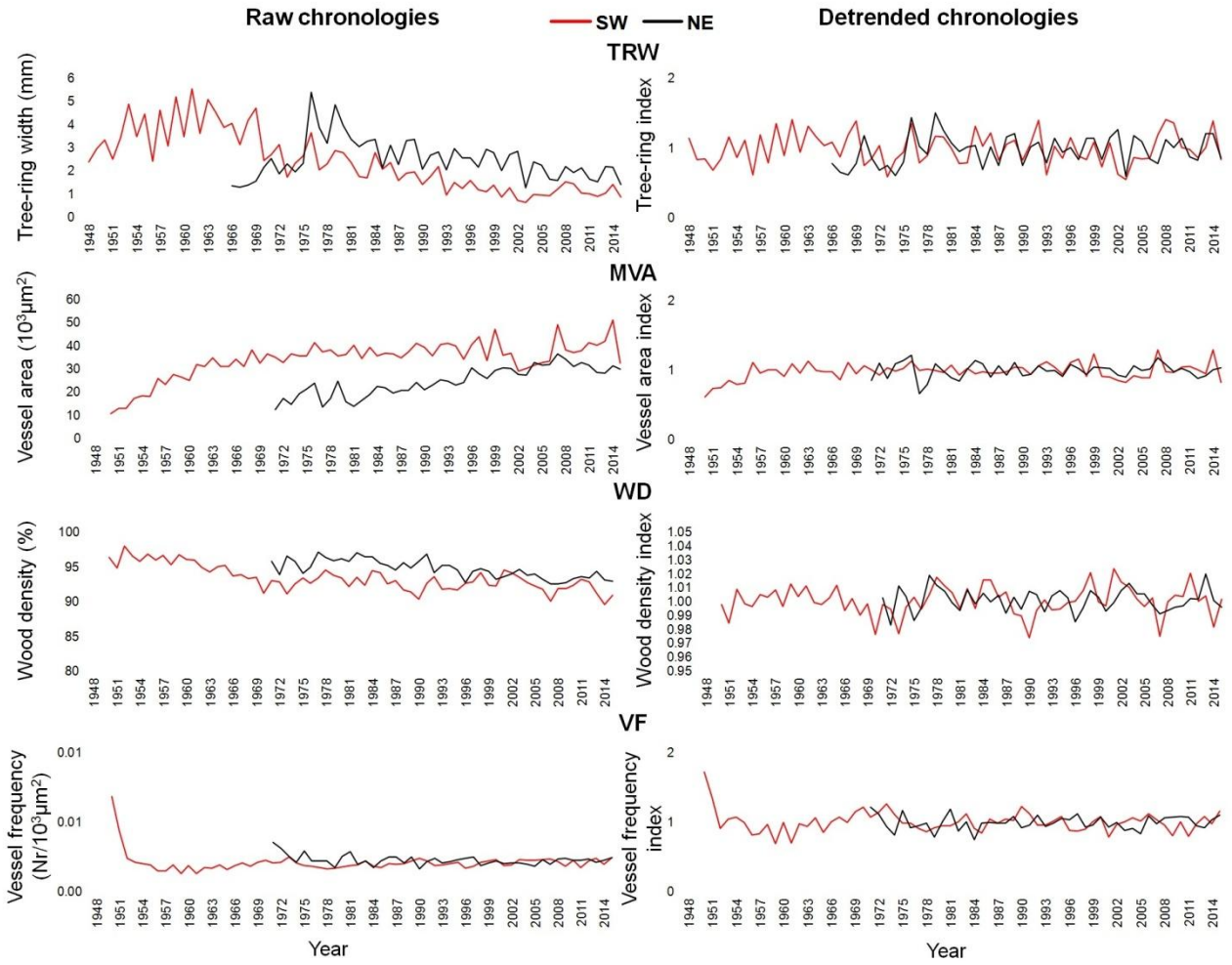

Fig. 2 Raw and detrended mean annual chronologies of tree-ring width (TRW), mean vessel area (MVA), wood density (WD) and vessel frequency (VF) of the two sites (SW in red, NE in black).

Table 3 IADF-related parameter values (**CP_{CA}**= referred to the common period aligned by cambial age; **SW**= South-West site; **NE**= North-East site).

	SW	NE
IADF frequency % (Nr rings with IADF/Nr rings tot)	21 (**CP_{CA}** = 27)	17
Mean stabilized IADF frequency ($f=Fn^{0.5}$ ± SE)	1.35 ± 0.24 (**CP_{CA}** = 1.76 ± 0.24)	0.85 ± 0.12
Mean IADF width (mm ± SE)	0.90 ± 0.08 (**CP_{CA}** = 0.6 ± 0.06)	0.86 ± 0.08
Mean vessel area ($10^3 \mu m^2$ ± SE)	9.89 ± 0.57 (**CP_{CA}** = 9.85 ± 0.57)	8.39 ± 0.9
Mean wood density (% ± SE)	98.09 ± 0.14 (**CP_{CA}** = 98.07 ± 0.15)	98.15 ± 0.17
Mean vessel frequency (Nr vessels/$10^3 \mu m^2$ ± SE)	0.0016 ± 0.0001 (**CP_{CA}** = 0.0016 ± 0.0001)	0.0020 ± 0.0002

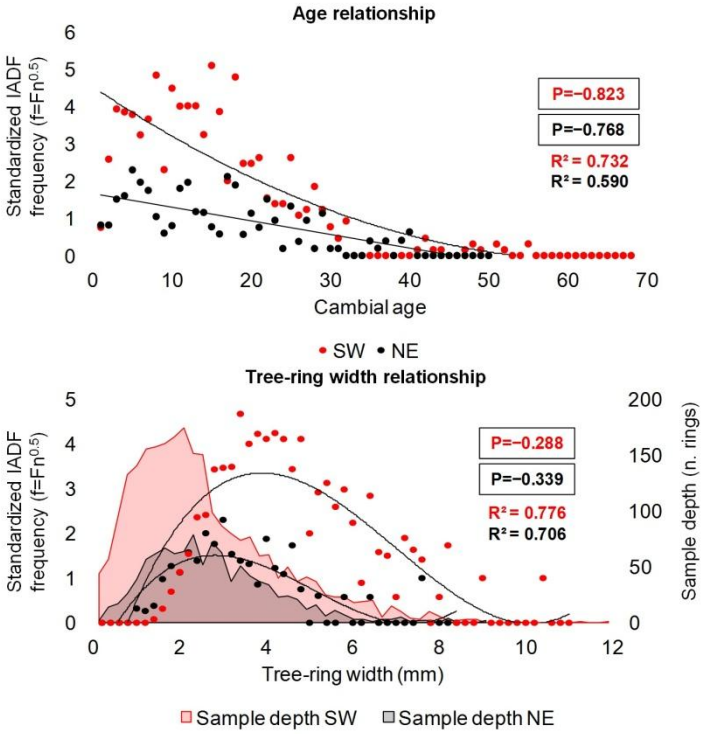

Fig. 3 IADF frequency chronologies aligned by cambial age and tree-ring width classes (SW in red, NE in black). P= Pearson product-moment correlation coefficient between each series and cambial age/tree-ring width, and R^2 of the fitted polynomial curves ($p<0.001$).

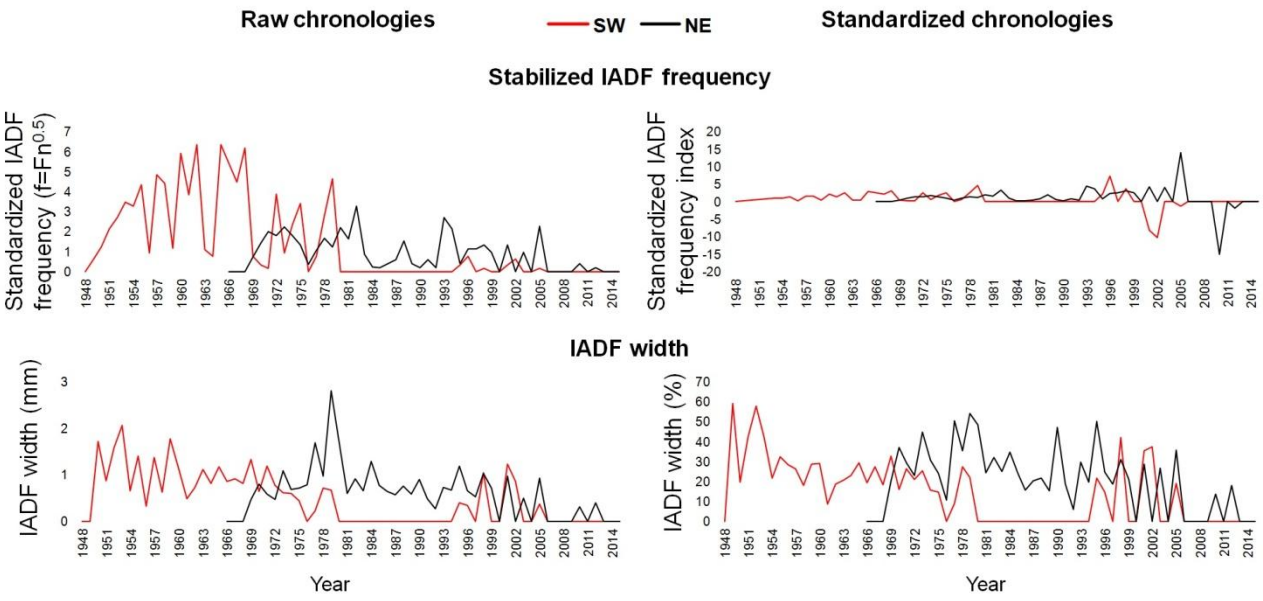

Fig. 4 Raw and standardized mean annual chronologies of stabilized IADF frequency and IADF width of the two sites (SW in red, NE in black).

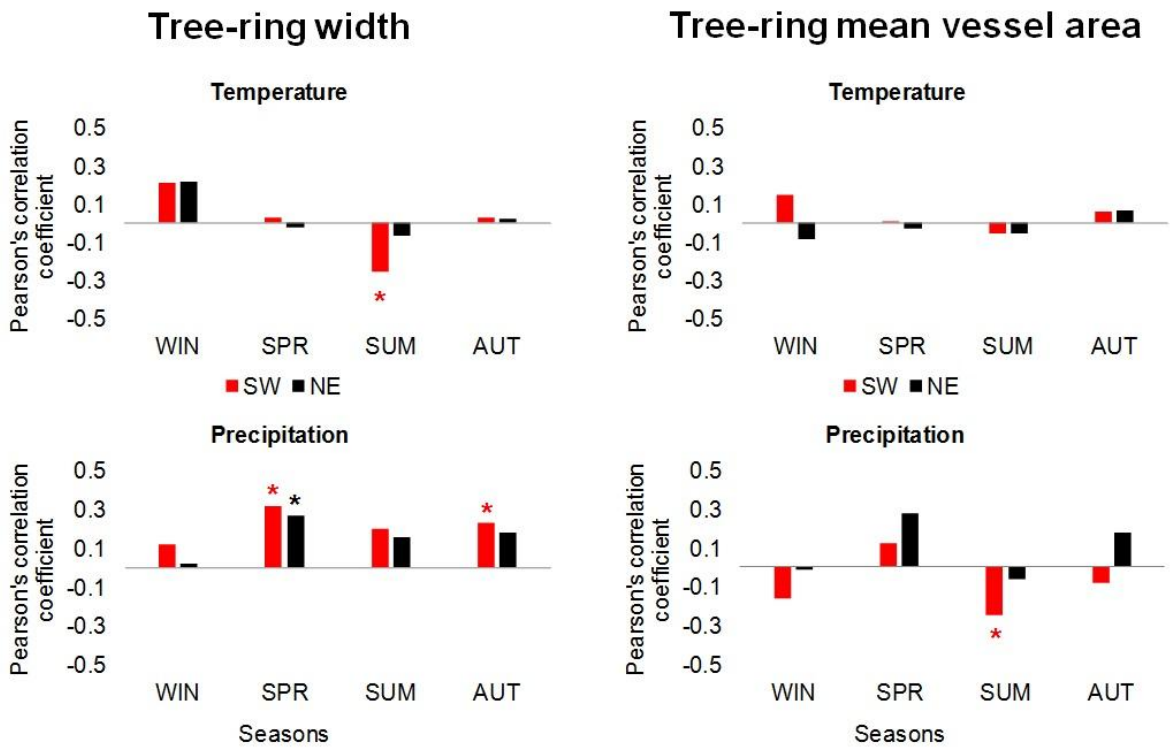

Fig. 5 Correlation function analysis between seasonally grouped mean temperature and total precipitation (WIN = from December of the previous year to March of the current year; SPR = from current March to current June; SUM = from current June to current September; AUT = from current September to current December) and tree-ring width and tree-ring mean vessel area with $P<0.05$ (SW in red, NE in black).

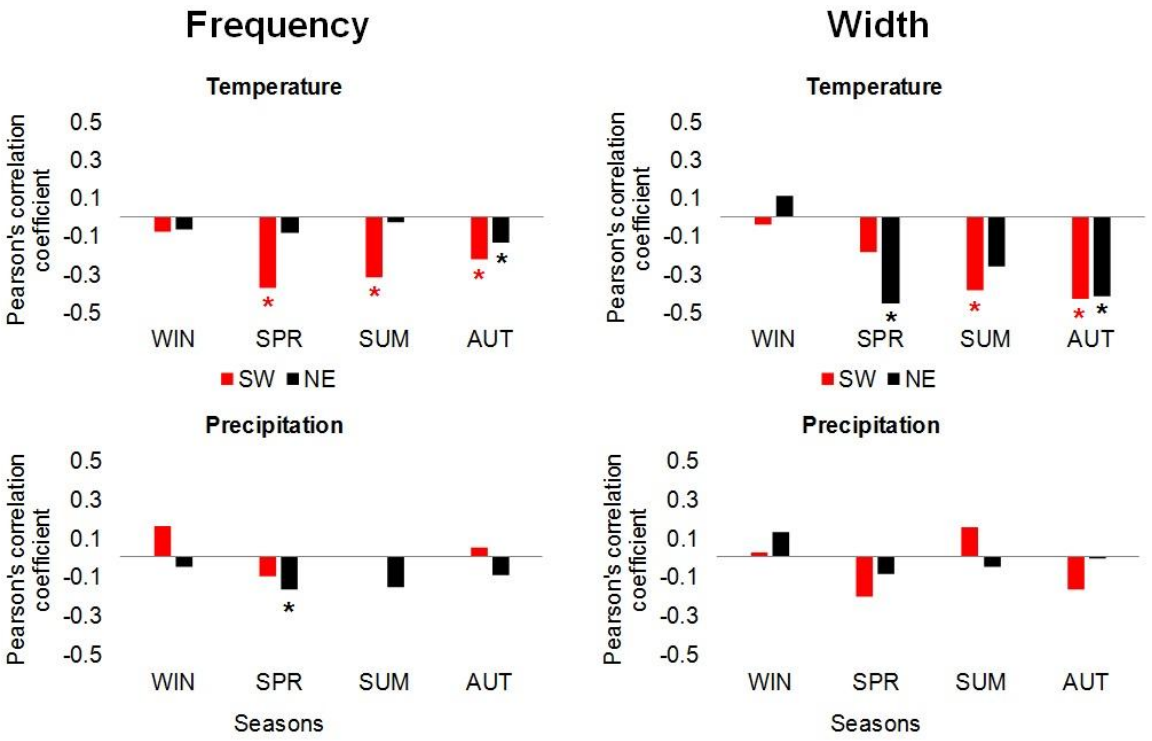

Fig. 6 Correlation function analysis between seasonally grouped mean temperature and total precipitation (WIN = from December of the previous year to March of the current year; SPR = from current March to current June; SUM = from current June to current September; AUT = from current September to current December) and IADF frequency and width with $P<0.05$ (SW in red, NE in black).

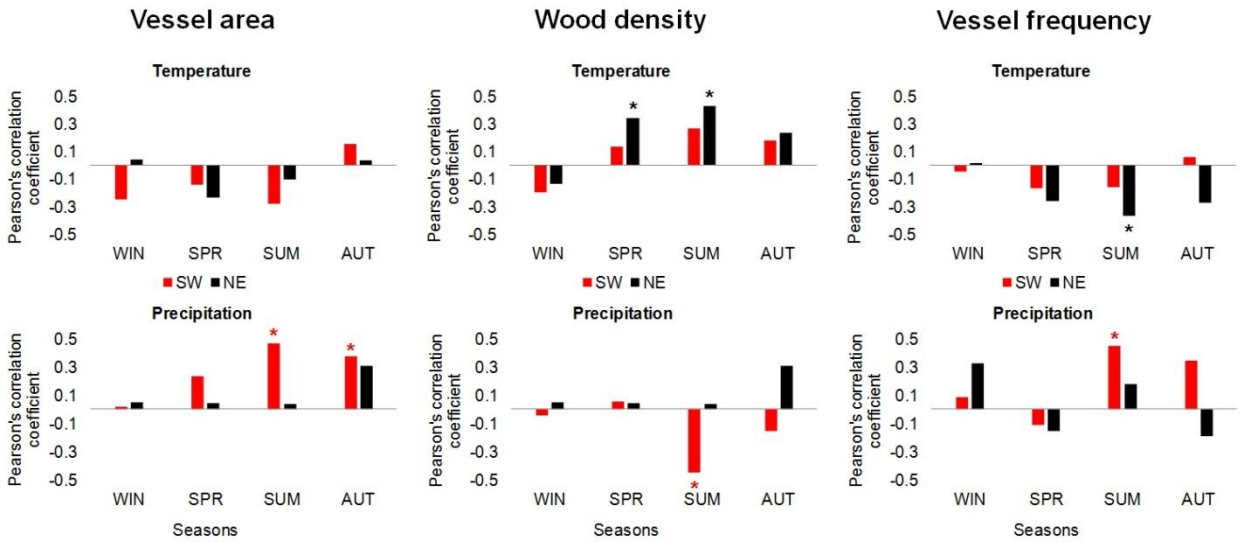

Fig. 7 Correlation function analysis between seasonally grouped mean temperature and total precipitation (WIN = from December of the previous year to March of the current year; SPR = from current March to current June; SUM = from current June to current September; AUT = from current September to current December) and IADF anatomical parameters (mean vessel area, wood density and vessel frequency) with $P<0.05$ (SW in red, NE in black).

4.9 Supplementary material

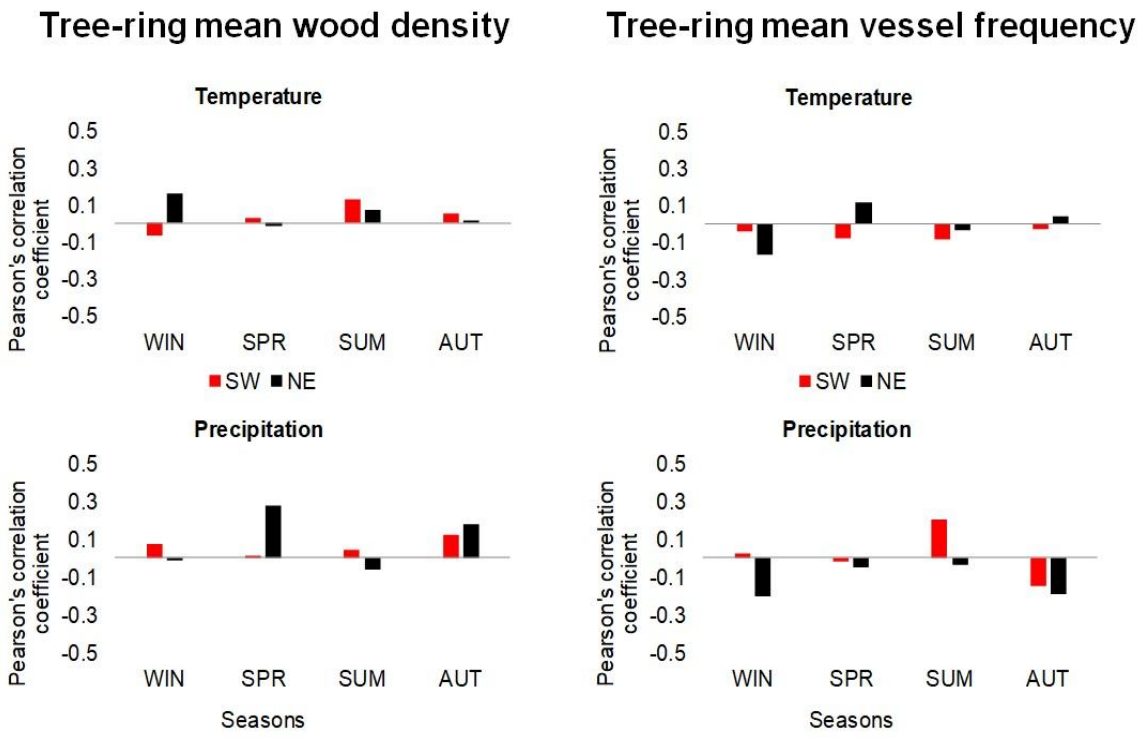

Fig. S2 Correlation function analysis between seasonally grouped mean temperature and total precipitation (WIN = from December of the previous year to March of the current year; SPR = from current March to current June; SUM = from current June to current September; AUT = from current September to current December) and tree-ring mean wood density and tree-ring mean vessel frequency with $P<0.05$ (SW in red, NE in black).

5. Pure *vs* mixed *Q. ilex* L. stands: stand-specific complementarity interactions and Intra-Annual Density Fluctuation frequency as indicators of adaptation to drought

5.1 Abstract

Climate response of tree-species growth may be influenced by intra- and inter-specific interactions. The different physiological strategies of stress response and resource use among species may lead to different levels of competition and/or complementarity, which likely change along sites and over time according to climatic conditions. Investigating the drivers of inter- and intra-species interactions under a changing climate is important when managing mixed and pure stands, especially in a climate change hot spot such as the Mediterranean basin. Intra-annual frequency of dry periods has been linked to the formation of peculiar anatomical traits in tree rings, the intra-annual density fluctuations (IADFs). Finding the link between IADF occurrence and stand productivity could be useful to add insights in their ecological role. In this study, we investigated the comparison of the temporal variation of cumulative basal area, intrinsic water use efficiency (WUE_i) and $\delta^{18}O$, and of IADF frequency within tree rings of *Q. ilex* growing in a pure and in a mixed stand with *P. pinea* in two study areas differing from stand characteristics in Southern Italy, in order to assess whether pure or mixed *Q. ilex* stands can be differently productive through time depending on climatic conditions and stand structure. The occurrence of a link between IADF frequency and stand structure under two microclimate conditions was also investigated. The occurrence of dryer climatic conditions triggered opposite complementarity interactions for *Q. ilex* growing with *P. pinea* trees in the two different study areas. Competitive reduction was experienced in the area with more soil water holding capacity, lower stand density and less slope, while competition occurred in the other one. The observed difference in productivity was reflected in a higher WUE_i due to a higher photosynthetic rate in the first while to a tighter stomatal control in the second mixed stand. At last, IADF frequency in *Q. ilex* tree rings seemed to be linked to stressful

conditions and could be interpreted as an adaptation aimed at avoiding dry periods, independently from stand productivity. Further study cases are needed to assess the influence of stand characteristics on complementarity interactions in Mediterranean *Q. ilex* mixed stands, and to allow the use of IADF occurrence as an indicator of species capability to avoid stressful conditions.

5.2 Introduction

Forest stand productivity is linked with climatic variability, stand structure, composition and density (Forrester, 2014). Whether pure or mixed stands can be more productive through time depends on abiotic factors such as climatic conditions, which in turn determine the availability of light and resources thus influencing stand development. In the mixed stands, together with stand development, there are changes in the dominance of species with different growth and physiological strategies (Forrester, 2015). Interactions between species could be ascribed to competition, with a negative effect of one species on the other, to competitive reduction, when inter-specific competition is less than intra-specific one because of a differentiation in resource use strategies, and/or to facilitation, with a positive effect of one species on the other (Forrester, 2014). Many studies have shown that mixed-species populations under stressful conditions are more productive than pure ones (Amoroso and Turnblom, 2006; del Río et al., 2014; Erickson et al., 2009; Pretzsch et al., 2013a,b), following the assumption of the stress gradient hypothesis (SGH) which suggests that, as condition become harsher, facilitation in spite of competition increases between species (Bertness and Callaway, 1994). However, this is not always the case and mixtures of species do not always show to be more adapted to climate constraints. Complementarity may show variations due to climatic factors which can be more or less intense depending on how different species react dealing with local characteristics such as stand density (Forrester et al., 2013), tree size (Forrester, 2015), site quality (Binkley, 2003; Coates et al., 2013; Dieler and Pretzsch, 2013; Pretzsch et al., 2010) and/or microclimatic differences (Lebourgeois et al., 2013). Changes in inter- and intra-

specific facilitation and/or competition interactions also depend on the different forest types, thus on the severity of climatic changes. Mediterranean area will be one of the regions most affected by the forecasted global warming (IPCC, 2017; Giorgi, 2006; Somot et al., 2008). The increased intra-annual frequency and duration of drought periods in Mediterranean areas may lead to changes in water use efficiency (WUE) which could facilitate one species in spite of another, i.e. faster growing trees in mixed stands could use more water than slower growing trees (Law et al., 2002; Schume et al., 2004), making them more sensitive to drought than less productive pure stands. Grossiord et al. (2014a,b) found that the stand-level $\delta^{13}C$ declined with diversity in temperate beech and thermophilous deciduous forests but not in hemi-boreal, mountainous beech and Mediterranean forests. Only few studies which compare complementarity effects between mixed and pure stands are available within the Mediterranean region. Grossiord et al. (2014c) found a negative impact of *Quercus petraea* (Mattuschka) Liebl. on *Quercus cerris* L. in a Mediterranean mixed stand under drought conditions in terms of transpiration, while Battipaglia et al. (2017) showed an increase in wood productivity and WUE in mixed stands of *Quercus robur* L. and *Alnus cordata* Loisel. in comparison with *Q. robur* pure stands, resulting from the positive N-fixation effect of *A. cordata*. Understanding what drives inter- and intra-species interactions under a changing climate is necessary when managing mixed and pure stands, since one of the priority in forestry is to acquire knowledge on the capability of different forest ecosystems to adapt to short- and long-term climatic variability (Brooker, 2006), especially in climate change hot spots such as the Mediterranean basin. *Quercus ilex* L. forests widely occur throughout the Mediterranean basin, both in pure stands or in mixed forests with Mediterranean pines such as *Pinus pinea* L. (Terradas, 1999), differing for light demand, root system and physiological strategies to drought response. Whether to maintain stand diversity or to push to the natural shifting to *Q. ilex* pure stands, is a still open question. Investigations have to be carried on the role that mixed stands exert in facilitate forest ecosystems to acclimate to forecasted climatic conditions. Intra-annual frequency of dry periods has been linked to the formation of peculiar anatomical traits in tree rings, the intra-annual density fluctuations

(IADFs), which are frequently found in tree rings of Mediterranean plants and could be used as indicators of specific climatic conditions (De Micco et al., 2016). The question whether they result from an hydraulic structure adjustment of trees to avoid stressful conditions or to take advantage of favorable ones is still open (Battipaglia et al., 2016a). Finding the link between IADF occurrence and facilitation and/or competition effects of different stands, which are linked to changes in climatic conditions, may be useful to add insights on the ecological role of these anatomical traits. In this study, we aimed to 1) compare the productivity of *Q. ilex* growing in a pure and in a mixed stand with *P. pinea* in two study areas differing for tree age, stand density and slope, and soil characteristics, 2) relate stand productivity to climatic factors, 3) find the link between wood anatomical recurrent traits such as IADFs, stand productivity and stand structure. In order to reach these aims, we investigated the temporal variation of cumulative basal area, intrinsic WUE (WUE_i) assessed through $\delta^{13}C$ in tree rings (Altieri et al., 2015; Battipaglia et al., 2016b; Moreno-Gutiérrez et al., 2012), and $\delta^{18}O$ of each pure stand in comparison with the relative mixed one. The combination of carbon and oxygen stable isotope analysis with tree-ring growth provides information about the ecophysiological processes experienced by trees in that specific year. In particular, the analysis of $\delta^{13}C$ combined with $\delta^{18}O$ allows to shed light on the different physiological strategies of stress response, suggesting which physiological trait concurred most to the variation in WUE, carbon uptake or water loss (Sheidegger et al., 2000). We hypothesize that: 1) *Q. ilex* productivity is higher in pure than in *P. pinea*-mixed stands, and such tendency changes according to stand characteristics; 2) temperature and precipitation influence *Q. ilex* productivity; 3) IADF occurrence is related to stand productivity.

5.3 Materials and methods

5.3.1 Study sites

The study sites are located within the Mediterranean Basin, in the Vesuvio National Park, southeast from Naples, southern Italy. A pure *Quercus ilex* population and a mixed *Q. ilex-Pinus pinea* population with comparable age, soil and stand characteristics were chosen on two opposite slopes of the Somma-Vesuvio volanic complex (Fig. 1). The sites of the two study areas, one in the southwest-faced "Tirone Alto-Vesuvio" Forest State Reserve (TP and TM sites) and the other on the northeast-faced Mount Somma slopes (SP and SM sites), differ by stand density, slope, aspect (Table 1) and soil characteristics. Both pure stands (TP and SP) are dominated by *Q. ilex* with an understory consisting in *Q. ilex* young trees and the sporadic presence of *Robinia pseudoacacia* L., a non-native invasive species. Equally, both mixed stands (TM and SM) are covered by *P. pinea* dominant trees with a *Q. ilex* understory and the sporadic presence of *R. pseudoacacia.* SP and SM sites are characterized by smaller trees and have higher stand density and slope than TP and TM sites (Table 1). Moreover, *P. pinea* trees of the SM site are taller than *Q. ilex* trees, while *P. pinea* and *Q. ilex* trees have a similar height at the TM site (Table 1). Total stand density is 33000 tree/ha in the pure while 19000 tree/ha in the mixed stand of the S study area, associated with a related canopy cover of 2.6 and 2.25, respectively, while the slope is 50 and 100% respectively. Instead, total stand density is 11000 tree/ha in the pure while 13000 tree/ha in the mixed stand of the T study area, associated with a related canopy cover of 1.2 and 1.69, respectively, while the slope is 20 and 0% respectively. Temperature and precipitation data from the nearest meteorological stations were fragmented and did not cover the whole study period; therefore they were interpolated and compared to the CRU TS3.23 gridded dataset at 0.5° resolution data (Harris et al., 2014). Since the correlation between the two data series was significant (supplementary material, Chapter 2, Fig. S1, Table S1, pag. 83), we considered the climate data from the CRU to be representative for the study sites. The climate is Mediterranean with dry summer and mild winter. Mean annual temperature and

precipitation of the period 1985-2005 selected for statistical analysis are 16.4°C and 710 mm, respectively (Fig. 1). The rainy month is November, with an average of 114.09 mm of precipitation, while the driest month is August, with an average of 24.42 mm of precipitation and the highest mean temperature of 25.22 °C. The lowest mean temperatures are recorded in January, with an average of 9.08 °C (Fig. 1). The aridity period lasts from the middle of May to the end of August.

5.3.2 Soil samples

Water content, available water capacity and water holding capacity were determined by taking 6 samples per study area (3 samples per site) and following standard procedures (USDA, Natural Resources Conservation Service, National Soil Survey Center, 1996). Undisturbed soil was collected with a PVC 10-cm cylinder with known weight filled up to the top. Each cylinder was sealed at the lower end with gauze and brought to the lab in a plastic bag without overturn it. The samples were then weighted (fresh weight) and placed in a few inches of distilled water for 48 hours to allow the water to rise by capillarity. Then, after the loss of the excess of water by percolation, samples were weighted to determine field capacity (the optimal condition of soil water content with saturated micropores). The soil of each sample was finally moved into aluminum bowls and placed in a stove at 105°C. Dry weight was determined when the samples reached a constant weight after drying.

5.3.3 Tree-ring growth

Two cores per tree were extracted at breast height from 20 dominant *Q. ilex* trees per site in the "Tirone Alto-Vesuvio" study area (T) and from 14 dominant *Q. ilex* trees per site in the Mount Somma study area (S). The number of cored trees per area is different because of the different availability of dominant trees. The increment cores were air dried, mounted on wooden supports

and sanded with progressively finer grades of sandpaper (up to 600 grit) to provide a flat surface in order to clearly identify tree-ring anatomical traits under magnification. Ring-width series were measured using a Leica MS5 stereoscope (Leica Microsystems, Germany) fitted with a LINTAB measuring system (Frank Rinn, Heidelberg, Germany), with a precision of 0.01 mm. Tree-ring width chronologies were visually cross-dated and statistically checked with the TSAP-Win (Time Series Analysis and Presentation; Rinntech, 2003) and COFECHA (Holmes, 1983) softwares. Once all the individual series had been verified, mean tree-ring chronologies were developed for each site. Chronology quality was assessed with the calculation of the expressed population signal (EPS), which indicates how well a chronology approximates the hypothetical perfect population chronology (Wigley et al., 1984), the mean RBAR, which is a measure of the common variance between individual chronologies, and the signal-to-noise ratio (SNR), which expresses their common signal strength (Table 2). Chronology quality assessment was implemented with the software R using the Dendrochronology Program Library (dplR; Bunn, 2008, 2010). In order to compare stand radial growth productivity, correctly dated tree-ring width chronologies were converted into tree basal area increment (BAI) chronologies: $BAI_t = \pi R_t^2 - \pi R_{t-1}^2$ (1), where R_t and R_{t-1} are the stem radius at the end and at the beginning of the annual increment, respectively, and BAI_t is the annual ring area at year t. Cumulative mean basal area was then calculated for each site summing the average basal area. BAI instead of ring-width time series were chosen because they reduce tree-size and age effect on growth trends, keeping the high and low frequency signals of tree-ring width series at the same time (Biondi and Qeadan, 2008; Tognetti et al., 2000).

5.3.4 Stable C and O isotope analysis

Five cores per site were chosen for isotopic analyses. Carbon and oxygen stable isotope analysis were conducted over the common period 1985-2005 for all the sites, where a change in wood productivity was found between pure and mixed stands in both the study areas. Tree rings were

manually split with annual resolution using a scalpel under a dissection microscope, and the derived samples of the five cores per species and per site were then pooled together in order to maximize sample size. Preliminary analyses showed that comparable results are obtained by using either whole wood or cellulose (Barbour et al., 2001; Borella et al., 1998; Korol et al., 1999; Loader et al., 2003; Verheyden et al., 2005; Warren et al., 2001; Weigt et al., 2015), thus we decided to proceed on whole wood, without any chemical pre-treatment, also to have enough wood material for isotope analyses also in the case of narrow rings. The samples were then milled with a centrifugal mill, weighted in silver capsules (aliquots of 0.8÷1.0 mg) and pyrolyzed at 1450°C (PYRO-cube, Elementar, Hanau, Germany) in order to determine the intra-annual $\delta^{13}C$ and $\delta^{18}O$ values of the obtained CO by a Delta Plus XP isotope ratio mass spectrometer (ThermoFinnigan MAT, Bremen, Germany) via a pyrolysis unit by a ConFlo III interface (ThermoFinnigan MAT). A correction of the resulted $\delta^{13}C$ values was made by measuring a subset of samples that covered the whole range of the expected $\delta^{13}C$ values via oxygen combustion, since the $\delta^{13}C$ signal obtained by pyrolysis is considered to be dampened because of "memory effects", compared to the most commonly used one obtained by oxygen combustion (Woodley et al., 2012). $\delta^{13}C$ values of the subset were measured via oxygen combustion with an EA1110 elemental analyzer (CE Instruments, Milan, Italy) coupled to a Delta-S isotope ratio mass spectrometer (ThermoFinnigan MAT). The correction used for the pyrolysis $\delta^{13}C$ data was the following: $\delta^{13}C_{corr} = 1.2526 \times \delta^{13}C_{pyro} + 5.0032$, where $\delta^{13}C_{corr}$ is the corrected final $\delta^{13}C$ value and $\delta^{13}C_{pyro}$ is the value measured by pyrolysis and corrected with internal standards. $\delta^{13}C$ values were further corrected for the Suess effect, which is a shift in the atmospheric concentrations of carbon isotopes due to increasing fossil-fuel derived CO_2 (Keeling, 1979). The corrected series were used for the subsequent statistical analyses.

5.3.5 WUE calculation from $\delta^{13}C$

WUE_i chronologies were calculated for each site using ^{13}C ratios measured in tree rings, since: $\delta^{13}C_{plant} = \delta^{13}C_{air} - a - (b - a) * (c_i/c_a)$ (2), where $\delta^{13}C_{air}$ is the carbon isotope ratio of atmospheric CO_2, a is the fractionation factor due to CO_2 diffusion through stomata (4.4 ‰), b is the fractionation factor due to the Rubisco enzyme during photosynthesis (27 ‰), c_i is the internal leaf CO_2 concentration, c_a is the atmospheric CO_2 concentration and $\delta^{13}C_{plant}$ is the carbon isotope ratio of plant organic matter. WUE_i was thus calculated following the formula reported by Ehleringer and Cerling (1995): $WUE_i = A/g_s = (c_a - c_i)/1.6$ (3), where A is the photosynthetic rate, g_s is the stomatal conductance and 1.6 is the ratio of diffusivity of water and CO_2 in the atmosphere. In particular, the following formula was used: $WUE_i = (c_a - c_i)/1.6 = [c_a - c_a(\Delta - a/b - a)]1/1.6 = c_a[(1-(\Delta - a/b - a))1/1.6]$ (2), where Δ is the carbon isotope discrimination which represents the difference between $\delta^{13}C_{air}$ and $\delta^{13}C_{plant}$, c_i is equivalent to $c_a[(\Delta - a)/(b - a)]$ (4), while c_a annual values were taken from the NOAA database (http://www.esrl.noaa.gov/, Mauna Loa station). The parameter Δ was calculated as: $\Delta = (\delta^{13}C_{air} - \delta^{13}C_{plant})/(1 + \delta^{13}C_{plant})$ (5). $\delta^{13}C_{air}$ values were taken from the ones estimated by McCarroll and Loader (2004) and the measured ones available online (http://www.esrl.noaa.gov/gmd/), while $\delta^{13}C_{plant}$ are the values measured in tree rings of our samples.

5.3.6 Complementarity calculations

In order to compare pure and mixed stand growth and to assess inter-species facilitation and competition interactions, an annual index of complementarity was calculated for the period 1985-2005 for each study area with the following formula (Forrester, 2015; Battipaglia et al., 2017):

$Complementarity\ (\%) = [(X_M - X_P)/(X_P)] *100$ (6),

where X is annual basal area, WUE_i or $\delta^{18}O$, M is related to mixed stands and P is related to pure stands. The index is positive when growth, WUE_i or $\delta^{18}O$ are higher in mixed than in pure stands, while negative when they are higher in pure than in mixed stands.

5.3.7 IADF frequency analysis

IADF occurrence was detected within the rings of all the dated cores under a stereomicroscope. IADFs were identified by detecting variations in cell lumen area, frequency and wall density different from the "standard" transition from earlywood to latewood of *Q. ilex* described in Wheeler (2011), as found in Campelo et al. (2007a) and defined in Chapter 2. Relative annual IADF frequency chronologies of the sites were calculated as the ratio between the number of cores with an IADF and the total number of cores for each year. Stabilized annual IADF frequency chronologies were then calculated according to Osborn et al. (1997) as $f = Fn^{0.5}$, where F is the relative IADF frequency value and n is the total number of cores for each year, in order to stabilize the variance and to overcome the issue of the changing sample depth over time. A percentage of IADF occurrence was calculated for each site as the number of rings with IADF on the number of total rings for the period 1985-2005.

5.3.8 Climate analysis

A Pearson's linear correlation function analysis ($P<0.05$) was implemented between cumulative mean annual BAI of the whole study period (1985-2005), of the period 1985-1996 and of the period 1997-2005, and temperature and precipitation data in order to investigate whether and what climate factor significantly influenced the concurrent change in wood productivity between pure and mixed stands of the two study areas. Temperature and precipitation data were seasonally grouped from December of the previous year to February of the next year, in order to cover all the growing season

which could last up to the first months of the next year in Mediterranean species (Cherubini et al., 2003; Vieira et al., 2015; Balzano et al., submitted). The analyses were performed using Excel©.

5.4 Results

5.4.1 Tree-ring growth, WUE_i and $\delta^{18}O$ trends

The mean annual BAI for the period 1985-2005 of stand SM was the lowest (1063.31 ± 311.26 mm^2, mean value ± standard error), followed by TP (1257.38 ± 360.67 mm^2), SP (1274.51 ± 298.34 mm2) and TM stand with the highest annual BAI (1665.08 ± 407.62 mm^2). Cumulative basal area chronologies covered the timespan from 1949 to 2014 at the two sites of the T study area, while the shorter timespan from 1966 to 2014 was covered by *Q. ilex* trees of the two sites of the S study area (Fig. 2). The cumulative basal area of the stands revealed an opposite shift in growth between pure and mixed stands from the year 1997 to 2014 between the two study areas (Fig. 2). A growth increase in the mixed compared to the pure stand was recorded in the T study area (Fig. 2a), while a growth increase in the pure compared to the mixed stand was shown in the S study area (Fig. 2b) from 1998 to the end of the chronologies. WUE_i was significantly higher for all the study period in the mixed (mean value = 84.15 ± 2.22 μmol mol^{-1}) compared to the pure stand (mean value = 78.04 ± 2.86 μmol mol^{-1}) in the T study area ($P<0.05$) (Fig. 3a), as well as for the S study area, but not for all the years (SM mean value = 79.91 ± 2.37 μmol mol^{-1}; SP mean value = 77.2 ± 2.9 μmol mol^{-1}) ($P<0.05$) (Fig. 3b). $\delta^{18}O$ values were similar between pure and mixed stands in both the study areas, while significantly higher values of $\delta^{18}O$ were found in both pure and mixed stand of the S (SP mean value = 25.75 ± 0.49 ‰; SM mean value = 25.87 ± 0.5 ‰) compared to the T (TP mean value = 25.04 ± 0.29 ‰; TM mean value = 25.05 ± 0.36 ‰) study area ($P<0.05$) (Fig. 3c, d).

5.4.2 Complementarity effects

After the first years of the study period, where no clear differences in basal area were shown until the 1992, the complementarity effect analysis pointed out the difference between facilitation/competition interactions of mixed and pure stands of the two study areas. From 1992 to 2005, *Q. ilex* wood growth was higher in the mixed than in the pure stand in the T study area, while it was higher in the pure than in the mixed stand in the S study area (Fig. 4). WUE_i was found to be substantially higher in mixed than in pure stands during all the study period in both the study areas (Fig. 5a). Finally, the wood of the mixed stand is more enriched in ^{18}O than the pure one in the S study area in most of the years, while an unclear pattern was shown for the complementarity index based on $\delta^{18}O$ values of the T study area (Fig. 5b).

5.4.3 IADF frequency

The highest percentage of occurrence of IADFs in *Q. ilex* rings for the period 1985-2005 was found at the two sites of the S study area. In particular, the highest one was recorded in the mixed stand of the S study area, followed by the pure one, then the mixed stand of the T study area followed by the pure one, which showed a very low IADF percentage of occurrence (Fig. 6).

5.4.4 Climate influence

Climate analysis with cumulative mean annual BAI of the whole study period didn't show significant correlations for any of the sites. The same lack of significant correlation was found for the analysis cumputed for the period 1985-1996. Instead, a significant influence of summer (from June to August) and autumn (from September to November) precipitation on cumulative mean annual BAI of the period 1997-2005 was found for all the sites, with higher Pearson's coefficient

values for autumn (TP = 0.82; TM = 0.82; SP = 0.85; SM = 0.85) than for summer precipitation (TP = 0.69; TM = 0.71; SP = 0.71; SM = 0.71) ($P<0.05$).

5.4.5 Soil samples

No significant differences in water content (WC), available water capacity (AWC) and water holding capacity (WHC) were found between the mixed and the pure stand of each study area. Significant differences were found between AWC and WHC of the two study areas, with both the parameter values higher in the T (AWC mean value = 33.07 ± 12.87 %; WHC mean value = 24.27 ± 7.33 %) than in the S (AWC mean value = 20.27 ± 5.33 %; WHC mean value = 16.71 ± 3.79 %) study area ($P<0.05$).

5.5 Discussion

The mean annual BAI for the period 1985-2005 as an indicator of the overall stand productivity showed the mixed stand of the T study area and the pure stand of the S study area as the most productive sites. More specifically, *Q. ilex* cumulative basal area increment used as a measure of comparison of stand wood productivity between sites, pointed out an opposite turn of the curves of the pure and the mixed stand between the two study areas starting from the year 1997, which is reflected in the temporal variability observed in the BAI-based complementarity indexes as well. Summer and autumn precipitation seem to have driven the observed shift in growth between pure and mixed stands, since climate correlations with cumulative BAI showed no significant influences until 1996, while summer and autumn precipitation affected tree growth of all the stands starting from 1997. After the year 1996 a decrease in both summer and autumn precipitation accompanied by an increase in temperature is recorded (supplementary material, Fig. S2), which translates into drier conditions that could have triggered the complementarity interactions. In the water-limited

Mediterranean climate, water availability could be the main driver for productivity of *Q. ilex* leading to changes in complementarity interactions, as the correspondence between the observed high values of the BAI-based complementarity index of the T study area and of summer precipitation in 1995 could show. Therefore, with the occurrence of drier climatic conditions, in the T study area an interaction effect of competitive reduction was experienced in the mixed stand with the aging of trees making *Q. ilex* more productive in the mixed than in the pure stand. This interaction of facilitation between different species growing in the same stand supports several studies which found mixtures more productive compared to monocoltures, being eased by many processes like the inter-specific differences in phenology and physiology that reduce competition for resources (Moore et al., 2011; Roupsard et al., 1999; Schwendenmann et al., 2015), or the use of different water sources (Schume et al., 2004; Schwendenmann et al., 2015). In our case study, it could be ascribed to different root systems with *Q. ilex* extracting water deeper in the soil than *P. pinea*, or more specifically, to the combination of an anisohydric species like *Q. ilex* which resists drought and an isohydric species as *P. pinea* which avoids it to conserve water (Chapter 2; Mayoral et al., 2015). Differences in *Q. ilex* productivity growing in pure and in mixed stands together with the occurrence of drier climatic conditions were also found in the S study area, even if with an opposite trend: competition outweighted any complementary effects in the mixed stand, making *Q. ilex* less productive than in the pure one. Tougher conditions of growth with higher density and slope, and a soil with a lower water holding capacity could have concurred to make *Q. ilex* more affected by *P. pinea* competitiveness in the S study area; moreover, stand density is in favor of *P. pinea* in SM while of *Q. ilex* in TM. This assumption would be in contrast with the stress gradient hypothesis (SGH) which suggests that facilitation in spite of competition increases between species when site conditions are harsher (Bertness and Callaway, 1994). However, it would instead agree with the resource-ratio theory described by Tilman (1985, 2007), which implies that inter-specific competition may be stronger where soil fertility and moisture is lower, as showed by Trinder et al. (2012) for grassland species and by Coates et al. (2013) for *Pinus glauca* associated with *Pinus*

contorta. To further support this theory, Hunt et al. (1999) found that facilitation effects decreased with increasing stand density in *Eucalyptus nitens* stand mixed with *Acacia dealbata* in Australia; Río and Sterba (2009) showed a lower growth in mixed than in pure stands of *Pinus sylvestris* and *Quercus pyrenaica* in Spain driven by forest density. The analysis of the WUE_i and the $\delta^{18}O$ together with their relative complementarity indexes, revealed that *Q. ilex* in mixed stands had a higher WUE_i at similar $\delta^{18}O$ in both the study areas, although with both the stands of the S study area more enriched in ^{18}O than the T one, which indicates that a tighter stomatal control has been carried on compared to the T study area stands, possibly linked to its dryer conditions with a soil characterised by less water holding capacity thus a higher vapor pressure deficit at the leaf level (Barbour et al., 2002; Roden and Ehleringer, 2000). However, the higher $\delta^{18}O$ in the S than in the T study area could be also due to the fact that trees growing at the S study area are younger and may rely on water from upper soil layers, compared to the trees of the T study area, which tend to be enriched in ^{18}O because evaporative isotopic franctionation decreases with soil depth (Dawson et al., 2002). A difference in WUE_i not associated with a difference in $\delta^{18}O$ indicates that the high WUE_i observed in *Q. ilex* trees of the mixed stands was due to a higher photosynthetic rate rather than a lower stamatal conductance (Scheidegger et al., 2000). The processes that improve light and nutrient availability or uptake which are linked with inter-specific differences in mixtures can enhance WUE enabling the plants to increase photosynthesis and make more efficient use of their water (Forrester, 2015). Kunert et al. (2012) and Schwendenmann et al. (2015) found a higher WUE in mixtures than in monocoltures of tropical plants due to complementary water use, as well as Forrester et al. (2010) showed an enhanced WUE due to increased N and P availability and light absorption in mixtures which increased photosynthesis in *Eucalyptus globulus* growing with *Acacia mearnsii*. A high WUE is generally associated with a high stand wood growth (Binkley et al., 2004; Binkley 2012), as we found for the mixed stand of the T study area; however, the higher WUE which was also found in the mixed compared to the pure stand of the S study area, did not lead to an increase in tree growth, in accordance to other studies showing the lack of coherence between the

two parameters for several Mediterranean species during drought periods (Battipaglia et al., 2013; Maseyk et al., 2011; Moreno-Gutiérrez et al., 2015; Peñuelas et al., 2011), or even warming-induced growth reductions in spite of increasing WUE_i (Granda et al. 2014; Linares and Camarero 2012; Peñuelas et al. 2008). Indeed, carbon resources may be allocated to other biomass systems such as roots (Dewar et al., 1994), considering that during drought periods carbon investments in the below ground growth are of higher priority than the above ground one (Kotzlowski and Palladry, 2002) in order to guarantee water taking (Saxe et al., 1998). The absence of an enhanced basal area in concomitance with high WUE_i in the SM stand, rather a decrease compared to the SP, could be explained by the tighter stomatal control that was shown with the $\delta^{18}O$-based complementarity index. A decline in tree-ring width at enhanced WUE_i can be caused by a reduced stomatal conductance due to increasing warming-related drought, as Brito et al. (2016) showed for *P. canariensis* in Spain. According to the observed cumulative BAI reduction, the less favorable growth conditions in the S study area, with a higher tree density and slope and a lower soil water holding capacity of the topsoil, could have concurred to intensify the drought-induced stomatal closure reducing transpiration in the mixed stand, at the price of reducing net assimilation rate, as Brito et al. (2014) showed for *P. canariensis* at a treeline site with low WHC. *Q. ilex* trees growing in the mixed stand of the S study area could have been more affected by competition given that *P. pinea* trees presence prevailed. Furthermore, young Mediterranean trees could be more sensitive to limiting climatic conditions than older ones (Brito et al., 2016; Rozas et al., 2009, 2013; Vieira et al., 2009; Zalloni et al., 2016), leading to the hypothesis that the younger *Q. ilex* trees of the S study area suffered from competition with *P. pinea* rather than benefited from facilitation. Thus the harsher growth conditions of the S study area affected more the inter- than the intra-specific competition, even if the SP stand had a higher total density than the SM one, but a lower slope. Coherently, WUE_i and $\delta^{18}O$-based complementarity indexes showed that competition prevaled on facilitation in the mixed stand of the S study area, where the higher WUE_i was however accompained by higher ^{18}O ratios compared to the pure stand, suggesting a tighter stomatal control

of *Q. ilex* mixed with *P. pinea*, which was not shown for *Q. ilex* alone. More IADFs were formed in the rings of *Q. ilex* trees growing in the S study area, where harsher growth conditions of higher density and slope and a soil with a lower water holding capacity were present, and a tighter stomatal control was shown. A higher frequency of IADFs in tree rings enriched in ^{18}O related to a site with dryer growth conditions compared to a wetter site was also found in *Erica arborea* L. tree-rings by Battipaglia et al. (2014), leading to link the formation of these peculiar wood anatomical traits to stressful conditions rather than to favorable ones. Furthermore, within the two study areas, more IADFs occurred in the related mixed stands compared to the pure ones: the high IADF occurrence could thus be also linked to the higher WUE recorded in the rings of *Q. ilex* growing at the mixed sites. A high WUE is often linked to the ability of a species to withstand water stress (Battipaglia et al., 2014), and interpreted as an adaptation to drought-prone environments (Raven, 2002). In this sense, IADF frequency in tree rings could be linked to the ability of a species to avoid stressful conditions, and to the maintenance of a positive carbon balance under dry conditions with a high WUE.

5.6 Conclusions

The differences showed between productivity of *Q. ilex* pure and mixed stands growing in two different study areas, highlighted the importance of local site conditions in inter- and intra-species interactions underlying the growth response to environmental variability. The occurrence of dryer climatic conditions have shown to trigger opposite complementarity interactions for *Q. ilex* growing with *P. pinea* trees in two study areas differing for soil water holding capacity, stand density and slope. Competitive reduction was experienced in the area with more soil water holding capacity, lower stand density and less slope, while competition occurred in the other one. The observed difference in productivity was reflected in a high WUE due to a high photosynthetic rate in the first while to a tight stomatal control in the second mixed stand. At last, IADF frequency in *Q. ilex* tree-

rings seemed to be linked to stressful conditions rather than to favorable ones, and could be interpreted as an adaptation aimed at avoiding dry periods, independently from stand productivity. Further case studies are needed to assess the influence of stand density, slope and soil water holding capacity on complementarity interactions in Mediterranean *Q. ilex* mixed stands, and to be able to use IADF occurrence as an indicator of species capability to avoid stressful conditions.

5.7 References

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5.8 Figures

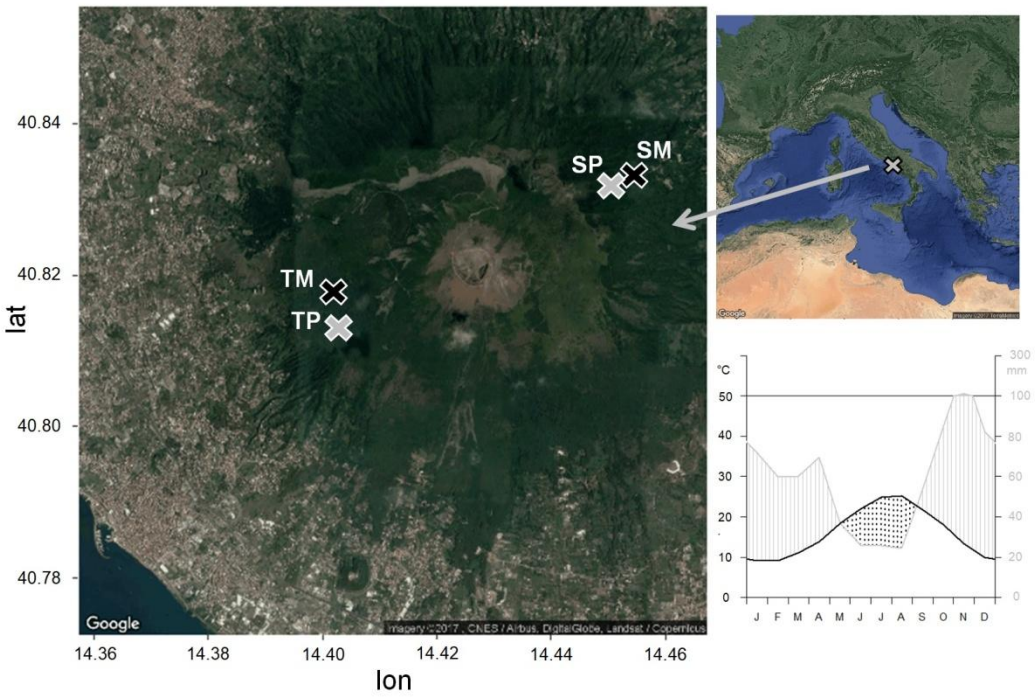


Fig. 1 Location of the study sites and climatic diagram of the period 1985-2005, from the CRU TS3.23 gridded dataset at 0.5° resolution data (Harris et al., 2014). Pure stands are in grey, mixed stands are in black.

Table 1 Coordinates, altitude and stand structure features of the study sites. TP and TM are the pure and the mixed stand, respectively, of the "Tirone Alto-Vesuvio" study area, while SP and SM are the pure and the mixed stand, respectively, of the Mount Somma study area.

	TP	TM	SP	SM
Latitude, Longitude (°)	40.49050 N, 14.24124 E	40.812909 N, 14.402956 E	40.49902 N, 14.27067 E	40.832987 N, 14.454074 E
Altitude (m a.s.l.)	528	505	669	569
Mean stem diameter (cm)	34	38	24	19
Mean tree height (m)	16	17	13	13
Q. ilex stand density (tree/ha)	11000	10000	33000	9000
Total stand density (tree/ha)	11000	13000	33000	19000
Canopy cover (Leaf Area Index - LAI)	1.2	1.69	2.6	2.25
Slope (%)	20	0	50	100
Mean *P. pinea* stem diameter (cm)	-	53	-	44
Mean *P. pinea* tree height (m)	-	17	-	16

Table 2 Dendrochronological characteristics of tree-ring width chronologies of the study sites. TP and TM are the pure and the mixed stand, respectively, of the "Tirone Alto-Vesuvio" study area, while SP and SM are the pure and the mixed stand, respectively, of the Mount Somma study area.

	TP	TM	SP	SM
EPS	0.99	0.98	0.9	0.92
RBAR	0.86	0.7	0.45	0.5
SNR	118.84	42.84	9.62	11.13

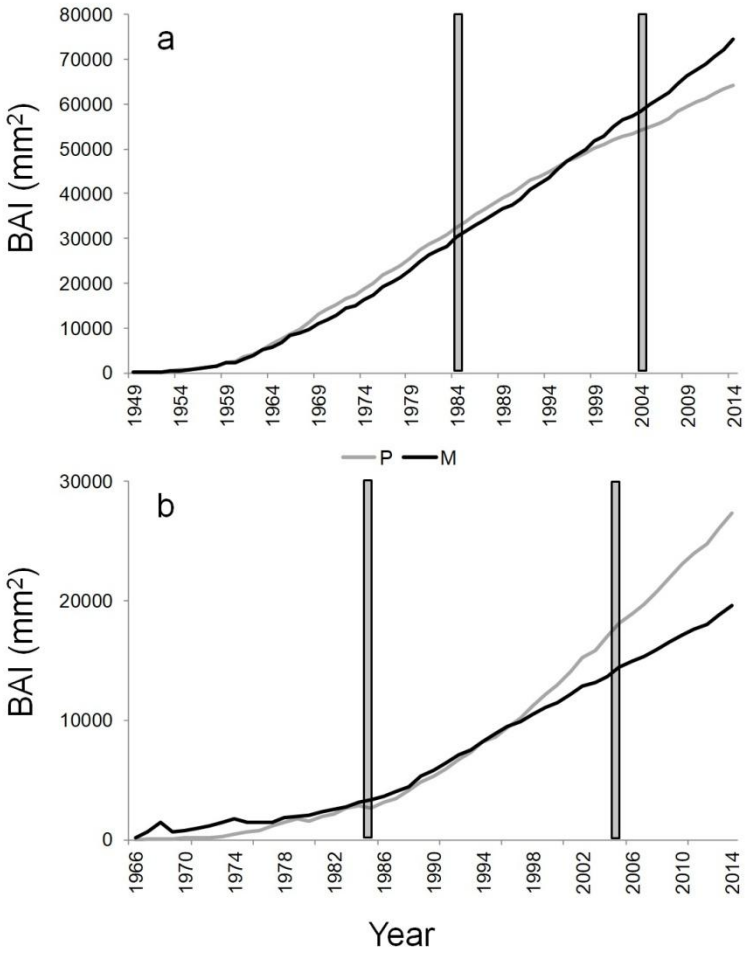


Fig. 2 Cumulative basal area of *Q. ilex* growing in pure (in grey) and mixed (in black) stands, in the "Tirone Alto-Vesuvio" (a) and in the Mount Somma (b) study area. The grey bars delimit the period chosen for isotope and statistical analysis.

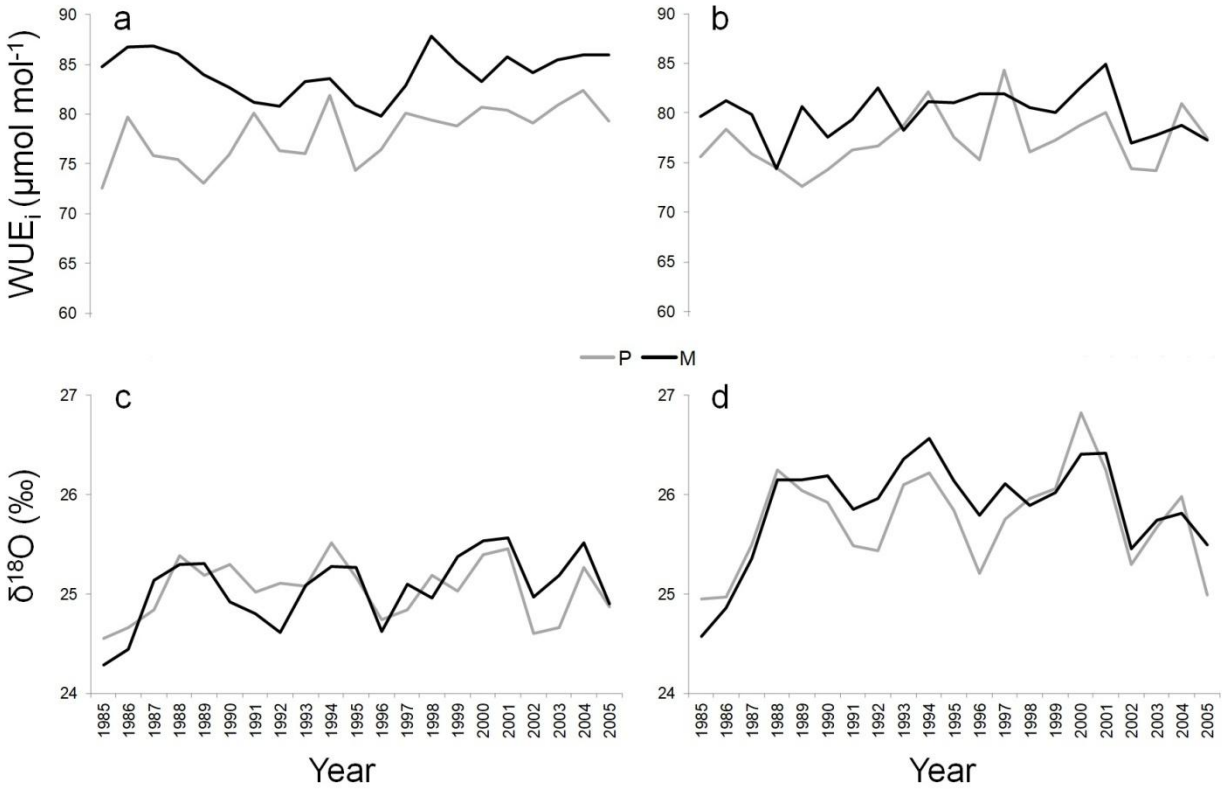


Fig. 3 WUE_i and $\delta^{18}O$ of *Q. ilex* growing in pure (in grey) and mixed (in black) stands for the period 1985-2005, in the "Tirone Alto-Vesuvio" (a, c) and in the Mount Somma (b, d) study area.

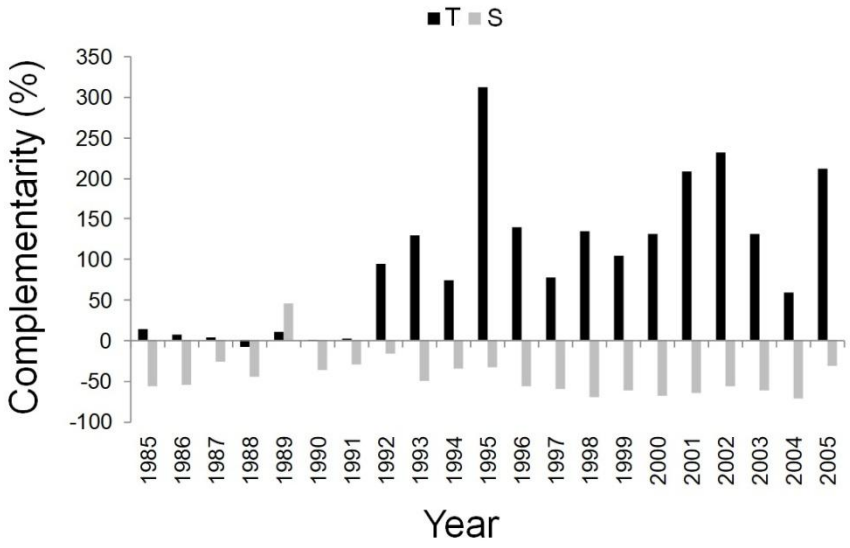


Fig. 4 Temporal dynamics of complementarity effect for the annual basal area of *Q. ilex* growing in the pure stand compared to *Q. ilex* growing in the mixed stand for the period 1985-2005, in the "Tirone Alto-Vesuvio" (in black) and in the Mount Somma (in grey) study area.

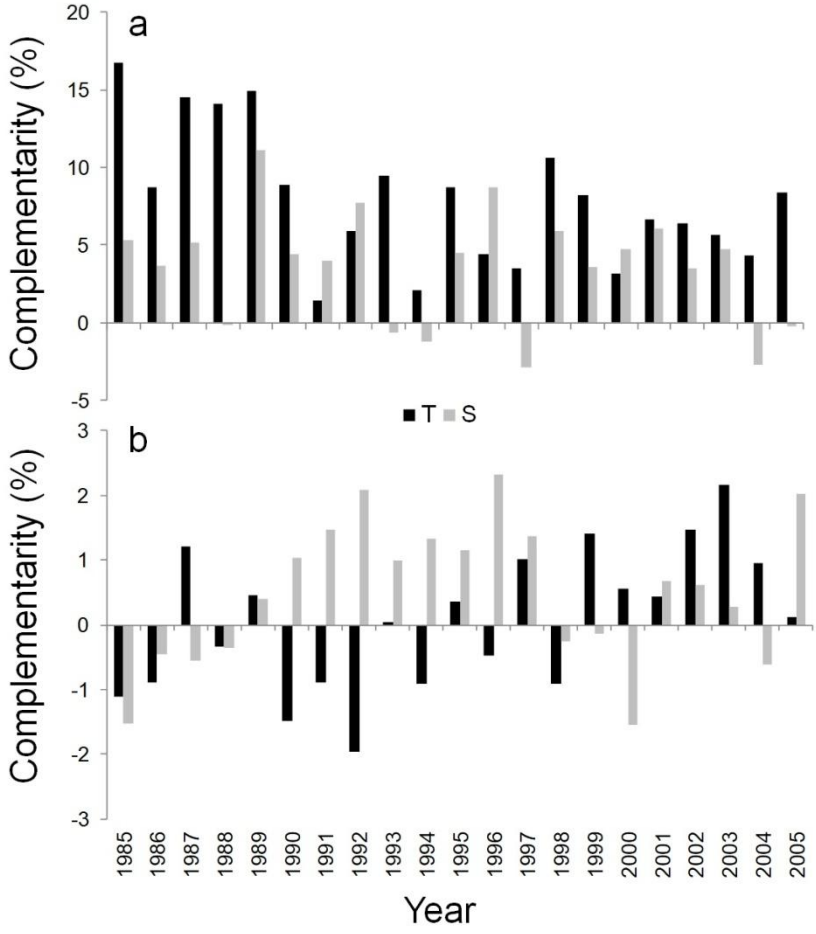
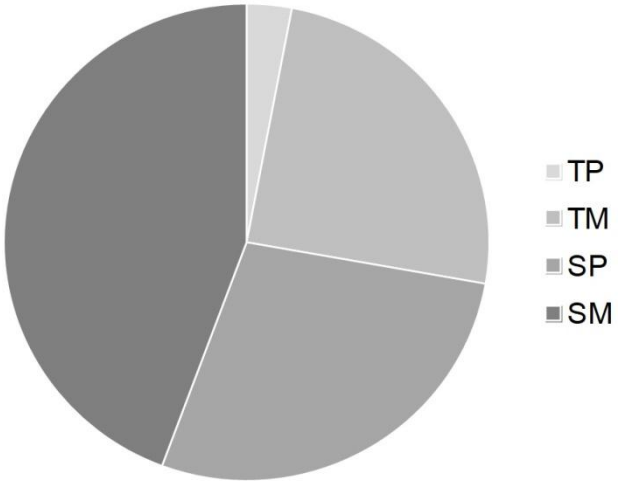


Fig. 5 Temporal dynamics of complementarity effect for WUE_i (a) and $\delta^{18}O$ o (b)f *Q. ilex* growing in the pure stand compared to *Q. ilex* growing in the mixed stand for the period 1985-2005, in the "Tirone Alto-Vesuvio" (in black) and in the Mount Somma (in grey) study area.



	TP	TM	SP	SM
IADFs (%)	1.79	14.76	16.61	26.41

Fig. 6 Percentage of IADFs calculated as number of rings with IADF on number of total rings for the period 1985-2005, for *Q. ilex* in the "Tirone Alto-Vesuvio" pure (TP) and mixed (TM) stand and in the Mount Somma pure (SP) and mixed (SM) stand.

5.9 Supplementary material

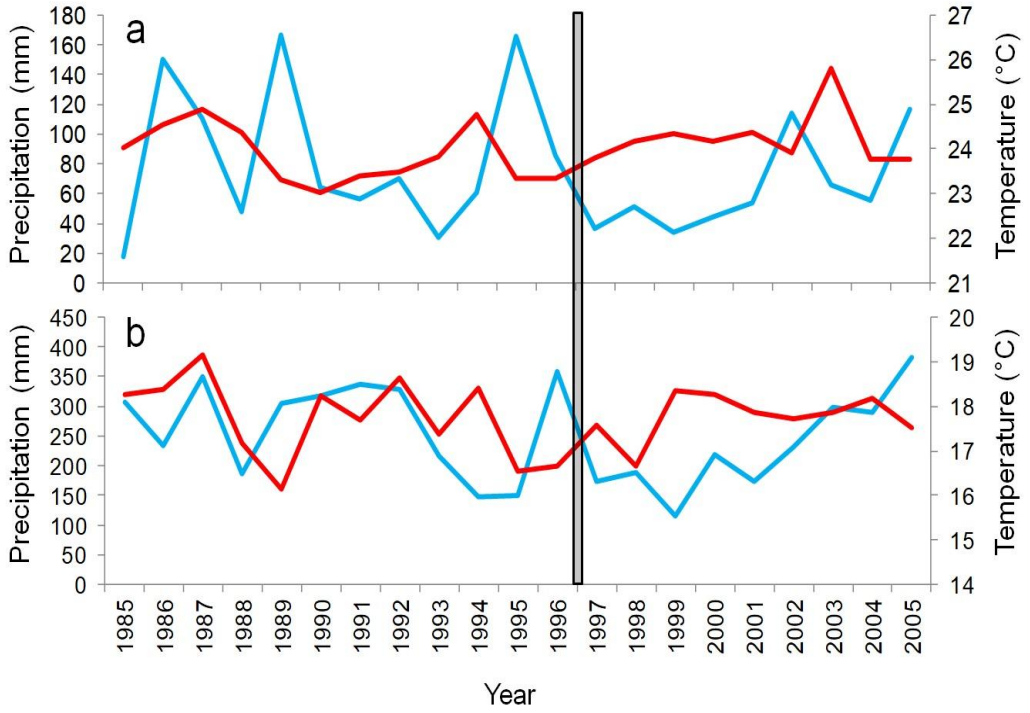


Fig. S2 Summer (from June to August) (a) and autumn (from September to November) (b) total precipitation (in blue) and mean temperature (in red) of the CRU TS3.23 gridded dataset at 0.5° resolution data (Harris et al., 2014) for the period 1985-2005. The grey bar indicates a decrease in both summer and autumn precipitation accompanied by an increase in temperature after the year 1996.

Here below the results of the additional analysis of the comparison between *Q. ilex* and *P. pinea* of the mixed stands (TM and SM) are shown. The methods to perform tree-ring growth and stable C and O isotope analysis, WUE_i and complementarity calculations, were the same reported for *Q. ilex* within this chapter. Dendrochronological indexes assessed the quality of the *P. pinea* tree-ring width chronologies (Table S1). *P. pinea* cumulative BAI was much more higher than *Q. ilex* in SM compared to TM (Fig. S3), coherently with the observed prevalence of competition between *Q. ilex* and *P. pinea* in the SM. The discussed high WUE_i of *Q. ilex* trees driven by a high photosynthetic

rate in the T study area while by a tight stomatal control in the S study area could be seen also in the higher WUE_i of *Q. ilex* compared to *P. pinea* associated with a higher $\delta^{18}O$ only in SM (Fig. S4). While, the hypothesis according to which the interaction of facilitation between the different two species growing in the T study area is eased by the use of different water sources with *Q. ilex* extracting water deeper in the soil than *P. pinea*, didn't find accordance with oxygen isotope analysis which showed similar values of $\delta^{18}O$ for the two species (Fig. S4c). The higher WUE_i of *Q. ilex* compared to *P. pinea* was due to a higher photosynthetic rate (Fig. S4a), which, anyhow, reflects its ability to withstand drought, since it helps in the maintenance of a positive carbon balance under dry conditions.

Table S1 Dendrochronological characteristics of *P. pinea* tree-ring width chronologies of the mixed study sites of the "Tirone Alto-Vesuvio" (TM), and of the Mount Somma study area (SM).

	TM	SM
EPS	0.99	0.98
RBAR	0.9	0.7
SNR	166.74	42.28

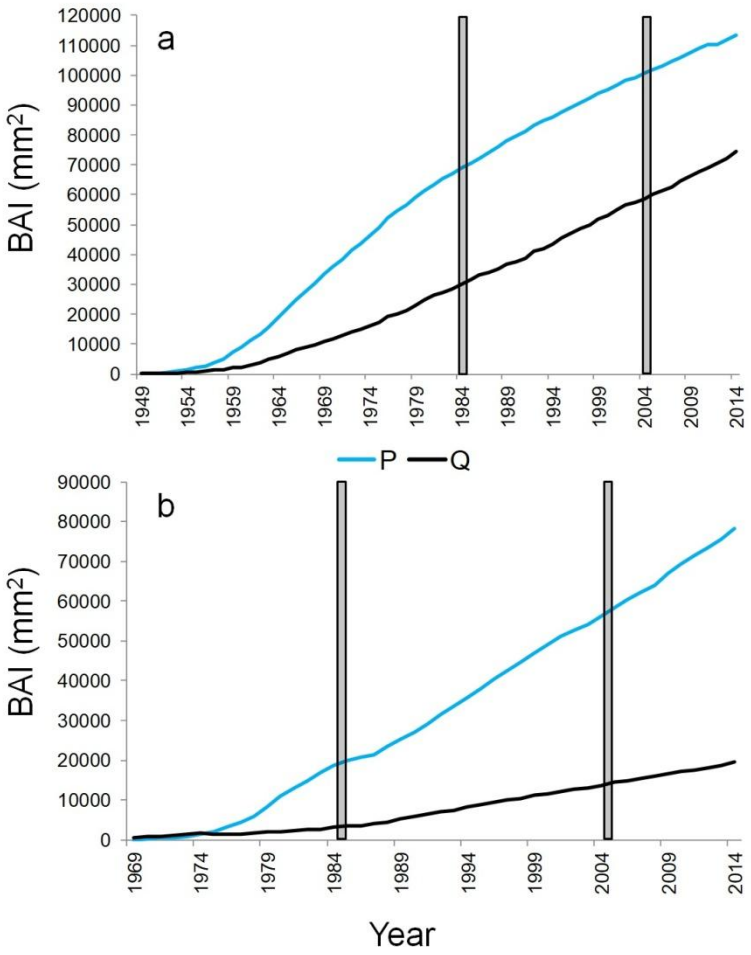


Fig. S3 Cumulative basal area of *P. pinea* (in blue) and *Q. ilex* (in black) growing in the mixed stands of the "Tirone Alto-Vesuvio" (a) and of the Mount Somma study area (b). The grey bars delimit the period chosen for isotope and statistical analysis.

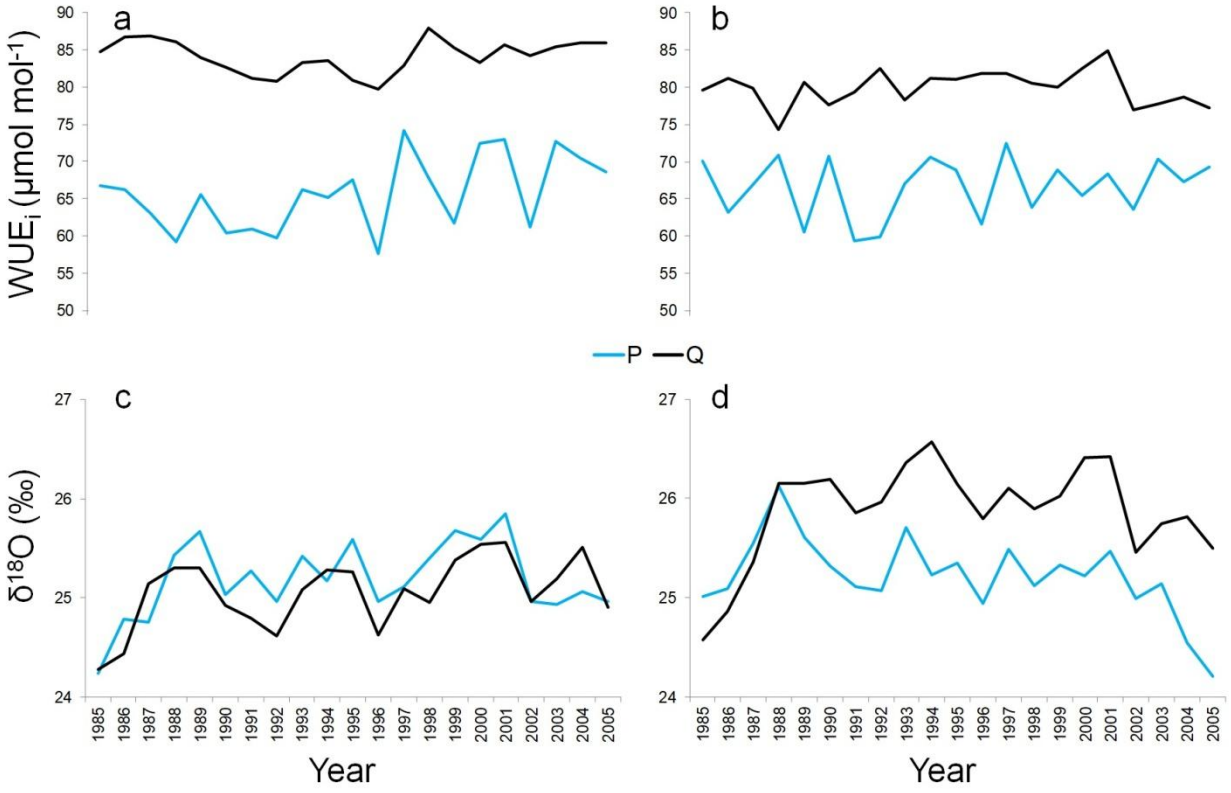


Fig. S4 WUE_i and $\delta^{18}O$ of *P. pinea* (in blue) and *Q. ilex* (in black) growing in the mixed stands of the "Tirone Alto-Vesuvio" (a, c) and of the Mount Somma study area (b, d) for the period 1985-2005.

6. A long tree-ring width chronology of *Q. ilex* L. from the Gussone Park of the Royal Palace of Portici, Southern Italy

6.1 Context

Q. ilex L. trees are widespread all over the Mediterranean basin and some of them, especially the ones growing within natural reserves or protected areas such as parks of royal palaces, are likely to be quite old. On June 16, 2014, a tornado caused extensive damage to the *Q. ilex* woodland "Parco Gussone", part of the Royal Palace of Portici (Reggia di Portici), Southern Italy, where is located the botanic garden "Orto Botanico di Portici" and of the Department of Agricultural Sciences of the University of Naples Federico II. The stem of several trees broke and many trees were completely uprooted. During the processes of securing the area, some cross sections of the fallen *Q. ilex* trees were taken in order to date them and to reconstruct their growth. The potential value of this species for dendrochronological and dendroclimatological studies has already been shown by some studies (Abrantes et al., 2012; Campelo et al., 2007, 2009; Corcuera et al., 2004; Gea-Izquierdo et al., 2009, 2011; Gutiérrez et al., 2011; Patón et al., 2009; Terradas and Savé, 1992; Zhang and Romane, 1991), but tree-ring dating of old *Q. ilex* trees is still a complex task which limits the number of studies using this evergreen Mediterranean species. One of the main problems in building *Q. ilex* tree-ring width chronologies is the frequent formation of intra-annual density fluctuations (IADFs) with unclear ring boundaries (Cherubini et al., 2003), which hampers the synchronisation of individual tree-ring series. However, thanks to the progress in identifying and characterising the IADFs, which took place during the last decade, what were previously considered as constraints in the application of dendrochronology are now considered as traits which may instead help crossdating tree-ring series (De Micco et al., 2016) as also demonstrated in the previous chapters of this thesis. In this appendix, we investigated whether it was possible to date the recovered *Q. ilex*

cross sections with the aim of building a long tree-ring width mean chronology which would help to date younger *Q. ilex* samples as those of the case studies previously showed in this thesis.

6.2 Study site

The study site is named "Parco Gussone – Bosco Superiore" (40°48'44.1972", 14°20'50.7408", with an altitude ranging between 90 to 15 m s.l.m.), and is located within the Park of the Royal Palace of Portici, along the south-western lower volcanic slopes of the Vesuvius and within the urbanized area of Portici, Naples, southern Italy (Fig. 1). The "Parco Gussone – Bosco Superiore" is an evergreen *Q. ilex* woodland with the presence of other Mediterranean broadleaved and conifer tree species such as *Celtis australis* L., *Cercis siliquastrum* L., *Ficus carica* L., *Fraxinus ornus* L., *Laurus nobilis* L., *Pinus halepensis* Miller, *Pinus pinea* L., *Quercus pubescens* Willd., *Robinia pseudoacacia* L., *Sambucus nigra* L., *Sorbus domestica* L., *Tilia americana* L. (Stinca and Motti, 2009). The establishment of the "Parco Gussone – Bosco Superiore" started in 1738 and *Q. ilex* seedlings from Sicily were used for the afforestation of the area.

6.3 Cross-section preparation and tree-ring width analysis

Twenty cross sections of *Q. ilex* trees taken at a stem height between the tree base and the breast height were available for the study. The sections were recovered from the available fallen trees throughout the "Parco Gussone – Bosco Superiore" area of 16.4 ha. Therefore, cross-sections from both dominant and dominated trees as well as plants internally injured or affected by knots were studied. Two segments per section were cut with a chainsaw following two opposite radii of the circumference, in order to be able to sand their surface. Therefore, the pith was present in almost all the segments, except for some of them where it was accidentally cut off. The segments were then air dried and sanded with progressively finer grades of sandpaper (up to 600 grit) to provide a flat

surface in order to clearly identify tree-ring boundaries under magnification. Five of the total available sections were excluded from the analysis because of the massive presence of disturbances such as scars and knots, which would have hampered the cross-dating. Therefore, fifteen cross-sections (thirty segments) were effectively used in the analysis. Two ring-width series per section (one per segment) were measured using a Leica MS5 stereoscope (Leica Microsystems, Germany) fitted with a LINTAB measuring system (Frank Rinn, Heidelberg, Germany), with a precision of 0.01 mm. Tree-ring width chronologies were visually cross-dated and statistically checked with the TSAP-Win (Time Series Analysis and Presentation; Rinntech, 2003) and COFECHA (Holmes, 1983) softwares. Chronology quality was assessed with the calculation of the expressed population signal (EPS), which indicates how well a chronology approximates the hypothetical perfect population chronology (Wigley et al., 1984), the mean RBAR, which is a measure of the common variance between individual chronologies, and the signal-to-noise ratio (SNR), which expresses their common signal strength. Chronology quality assessment was implemented with the software R using the Dendrochronology Program Library (dplR; Bunn, 2008, 2010). A mean tree-ring chronology was developed. The cross-dating tool of the TSAP-Win software was used to check the correspondence between the mean tree-ring chronology of "Parco Gussone" and all the *Q. ilex* mean chronologies of the sites studied in this thesis, since they are located about 10 km away and have the same climate conditions. The analysis was computed with the calculation of the Gleichläufigkeit (Glk) value which is a measure of the year-to-year agreement between the interval trends of two chronologies based upon the sign of agreement, usually expressed as a percentage of cases of agreement (Schweingruber 1988).

6.4 Results and discussion

The cross-dated and statistically checked tree-ring width individual series showed to have a high variability in age and ring width (Fig. 2). The relatively built mean annual chronology covered the

period 1823-2013, with an age of 190 years, and showed considerably narrow tree-ring widths, never reaching 3 mm (Fig. 3, Table 1). The validation indexes had low values, the EPS in particular was lower than the critical value of 0.85 (Table 1). *Q. ilex* has a ring to semi-diffuse-porous wood with multiseriate rays that can disturb the fine distinction of tree-ring boundaries, which, in addition, often show a sinusoidal tangential shape (Paton et al., 2009). Apart from these species-related difficulties in ring identification, another problem that we encountered was the very narrow rings with no clear boundaries at the external area of the oldest trees, which made the measurements complex (Fig. 4a). However, the availability of younger trees which presented wider rings helped to solve this problem, since their more clearly identifiable ring boundaries allowed to develop the chronologies and compare them to the older tree ones helping correct dating. Instead, the recurrent presence of disturbances such as scars and/or knots lead to distorted or abnormally wide/narrow ring periods (Fig. 4b, c, d), and could have included tree-ring width signals in the chronologies which would have obscured the common growth pattern. Furthermore, discontinuous rings along the circumference of the sections (wedging rings) were commonly found (Fig. 4e), hampering the process of dating. The high variability showed in the chronologies, which would have lowered the EPS value, could be also due to the fact that the studied trees were both dominant and dominated, recording possible periods of release and/or suppression not related to the common growth pattern.

Despite the encountered problems and the derived implications for the measured chronologies, the mean tree-ring chronology of "Parco Gussone" that we present here in this appendix, successfully cross-dated with all the *Q. ilex* mean chronologies of the study sites of this thesis, as demonstrated by the Glk values shown in Figure 5. Moreover, the majority of the curves showed the same trend in more than one year which is considered as a pointer for some common extreme event such as volcanic eruptions of the nearby Vesuvius, showed by the negative peaks of tree growth recorded in 1900 and 1929 (Nazzaro, 2000; Ricciardi, 2009).

In conclusion, this preliminary study helped us in dating all the younger *Q. ilex* samples used in the analyses showed within the chapters of this thesis, and lay bases for a more accurate dendochronological work on the "Parco Gussone" area. Next step would be coring only dominant trees in order to have a cleaner signal, taking care to sample trees without evident anomalies such as deformed stumps or knots. Working with cores instead of sections would be more difficult for these old trees, but no other cross-sections are available. However, once the validation indexes are sufficiently high and the mean chronology is enough representative of the entire population, it could be used to compute climate analysis in order to use it for archaeological dating as well as for aging old *Q. ilex* trees around the Mediterranean and for studying ecological and climatic issues.

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6.6 Figures

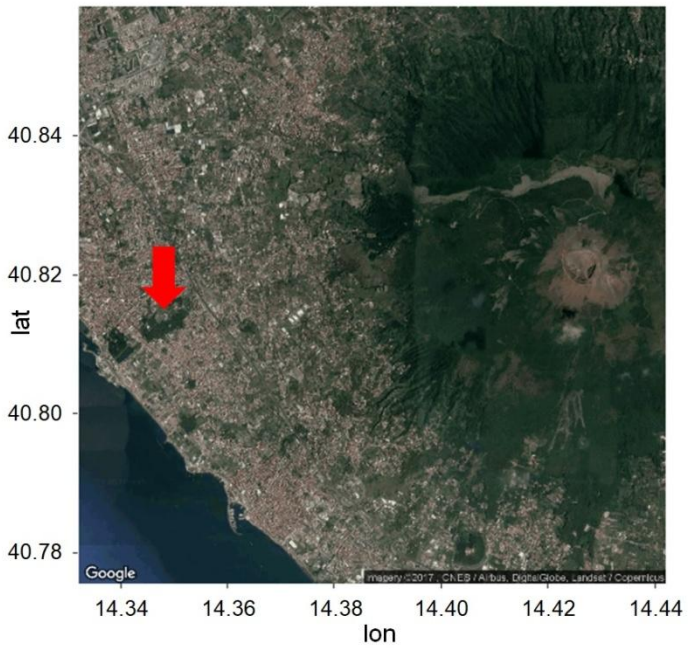

Fig. 1 Location of the study site, pointed by the red arrow. (Kahle and Wickham, 2013)

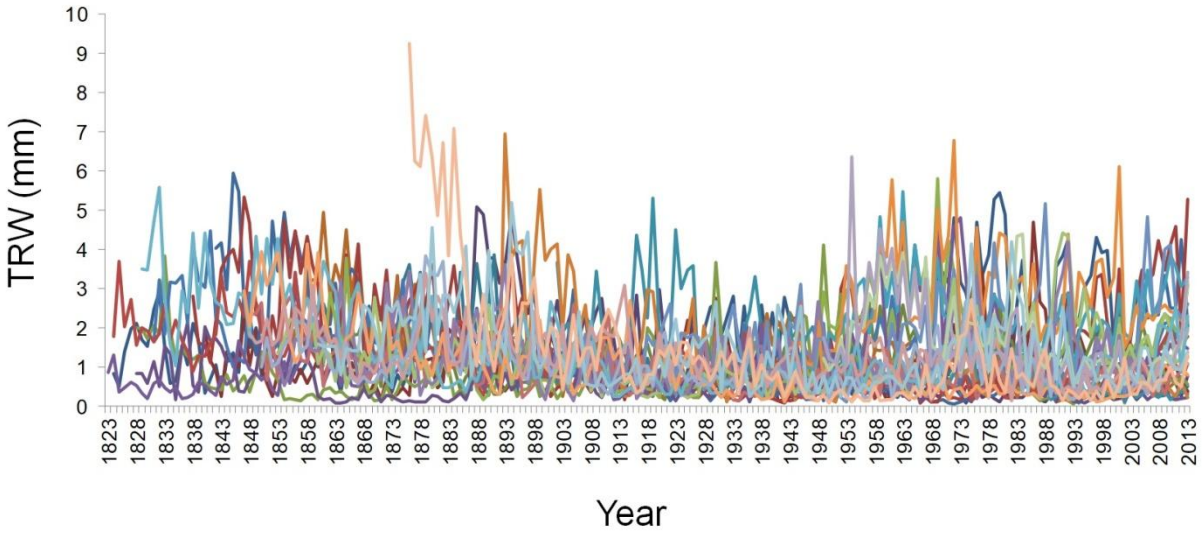

Fig. 2 Individual tree-ring width series of all the measured segments of the cross-sections of the "Parco Gussone" forest (**TRW**=Tree-Ring Width).

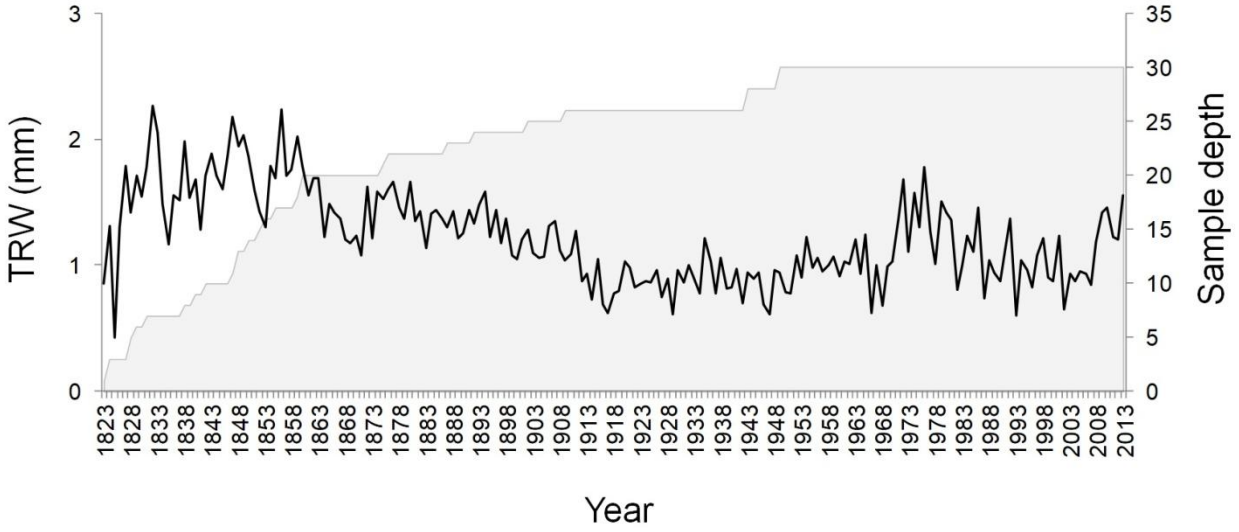

Fig. 3 Tree-ring width mean annual chronology of "Parco Gussone" woodland (**TRW**=Tree-Ring Width). In grey, the number of measured samples per year.

Table 1 Descriptive features and quality assessment indexes of the tree-ring mean annual chronology of "Parco Gussone" (**TRW**=Tree-Ring Width).

Timespan	Length	N samples	N trees	Mean TRW (mm±SD)	Max TRW (mm)	Min TRW (mm)	EPS	RBAR	SNR
1823-2013	190	30	15	1.22±0.36	2.27	0.42	0.77	0.12	3.29

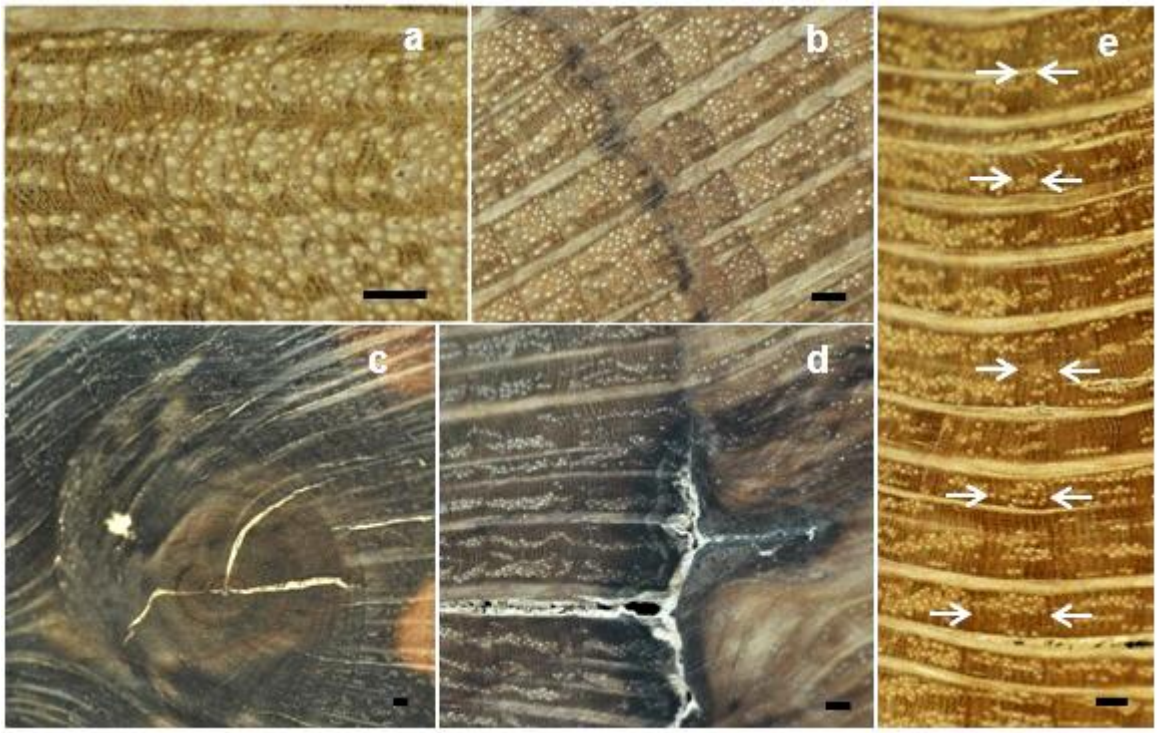

Fig. 4 Very narrow rings (a), scars (b, d), knots (c) and wedging rings (e) commonly found within the studied *Q. ilex* cross-sections. The white arrows indicate the borders of a tree ring which shrinks along the circumference until becoming a wedging ring. Bars: 1 mm.

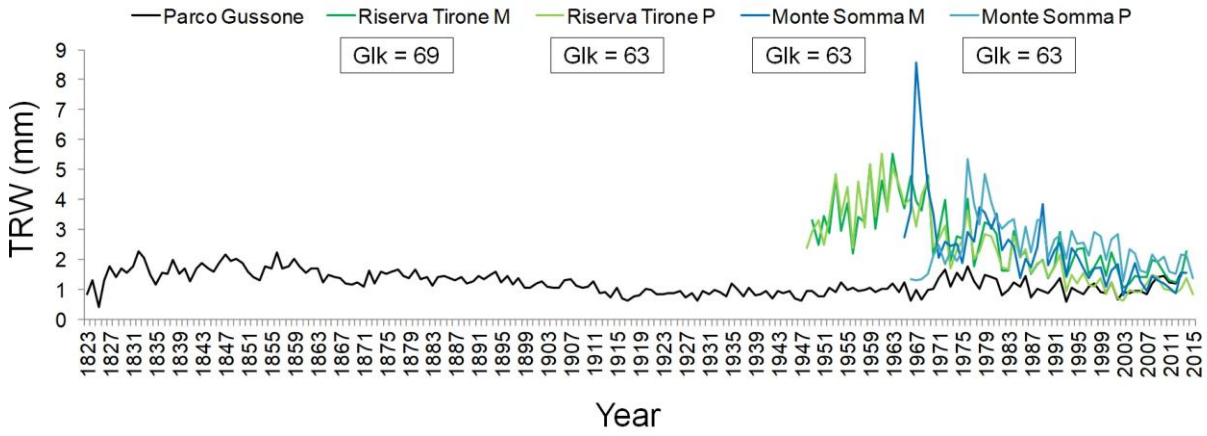

Fig. 5 Tree-ring width mean annual chronology of "Parco Gussone" and of all the other *Q. ilex* study sites of the thesis (**TRW**=Tree-Ring Width). The Glk values of the comparison between each mean chronology with the "Parco Gussone" one are shown.

7. General conclusions

The multidisciplinary approach applied in this thesis allowed finding a link between structure and function of Intra-Annual Density Fluctuations (IADFs) in tree rings of Mediterranean species in the case of both *Quercus ilex* L. and *Pinus* spp., and identifying the main climatic factors triggering tree growth. The combination of results from dendroecology, quantitative wood anatomy, stable isotope analyses, together with the analysis of their relations with climatic factors, allowed reaching all objectives stated in the study aims.

Tree-ring age needs to be taken into account when analyzing IADFs-climate relationships because it plays an important role in IADF formation as we found for both Mediterranean pines (Chapter 1) and *Q. ilex* (Chapter 3). Although Mediterranean pines and *Q. ilex* develop different types of IADFs, the highest frequency of IADFs was found in a common interval of tree rings in the two types of wood (Chapter 1, 3). Their occurrence was found mainly at the end of ring width, appearing as a band of earlywood-like cells within the latewood in Mediterranean pine tree rings while as an extra-growth band of wood located after the latewood in *Q. ilex* tree rings. A detrending method based on the age-dependance of IADF frequency chronologies was tested on a large tree-ring network of Mediterranean pines and then performed in the subsequent chapters. The existence of a common climatic driver for the formation of IADFs in Mediterranean pines was found, which was identified in autumn precipitation after summer drought, highlighting the potential use of IADF frequency as a proxy for climate reconstructions (Chapter 1). Our study allowed the anatomical and isotopical characterization of IADFs in *Q. ilex* and *Pinus pinea* L. coetaneous trees growing in the same Mediterranean stand; results suggested that IADF period of formation is during autumn months for both the species (Chapter 2). The occurrence of IADFs was found to reflect the more anisohydric/isohydric physiological reactions to the varying environmental conditions of *Q. ilex* and *P. pinea*, respectively: an increased stomatal conductance was associated to a safer wood at IADF level in *Q. ilex* while a tighter stomatal control was associated to a more efficient wood at IADF

level in *P. pinea*. Therefore, our results suggest that IADFs could be used as indicators of species-specific responses to intra-annual climate fluctuations. Moreover, the assessment of the influence of climate on IADF occurrence helped attempting predictions about species responses to future climate changes: in a climate scenario of a mean temperature rising and a decreasing in the precipitation pattern, *Q. ilex* would form less IADFs while *P. pinea* would be more prone to form IADFs (Chapter 2, 3). Indeed, the temperature increase in the Mediterranean area would affect cambial growth in *Q. ilex* likely inducing an earlier stop, not allowing the autumnal secondary re-growth which would lead to the formation of IADFs. Climatic correlations with anatomical traits of the IADFs in tree rings of *Q. ilex* helped distinguishing between different predominant climatic drivers of two sites on opposite slopes. Summer and autumn precipitation lead to the formation of IADFs with a more efficient wood in South-faced trees, with larger and more frequent vessels than trees from North-faced slopes. Spring and summer temperature influenced the anatomical structure of IADFs of North-faced trees, leading to a strategy more conservative than South-faces trees, with a denser wood also due to less frequent vessels (Chapter 3). The differences we found between sites could be ascribed to differences in aspect, soil water holding capacity and stand density. Therefore, the impacts of intra-annual climate fluctuations on IADF formation can vary depending not only on the species, but also on local site conditions. Local site conditions proved to be involved also in inter- and intra-species interactions underlying wood growth response to environmental variability (Chapter 4). The occurrence of dryer climatic conditions have shown to trigger opposite complementarity interactions for *Q. ilex* growing with *P. pinea* trees in two study areas differing for soil water holding capacity, stand density and slope. Competitive reduction was experienced in the area with more soil water holding capacity, lower stand density and less slope, while competition occurred in the other one. The observed difference in productivity was reflected in higher water use efficiency (WUE) due to a higher photosynthetic rate in the first while to a tighter stomatal control in the second mixed stand. At last, IADF frequency in *Q. ilex* tree rings seemed to be more linked to stressful conditions rather than to favorable ones, and could be interpreted as an adaptation aimed at

avoiding dry periods, independently from stand productivity (Chapter 4). Finally, the 190 years old *Q. ilex* tree-ring width mean chronology which we built on cross sections of trees fallen due to a natural storm in the *Q. ilex* woodland "Parco Gussone", part of the Royal Palace of Portici (Reggia di Portici), Southern Italy, helped us to date all the younger *Q. ilex* samples of the case studies which we present in this thesis and laid bases for a more accurate dendochronological analysis (Chapter 5).

Mediterranean pines and *Q. ilex* populations showed to be newsworthy study areas for the investigation of plant intra-seasonal responses to Mediterranean climate, where precipitation and temperature fluctuations, which lead to the succession of different periods of water deficit and surplus during the year, affect wood growth. The study cases reported in this thesis provide useful information on the ecological behavior and vulnerability of the species, especially of *Q. ilex*, leading to an improvement of the implementation of management options in such a climate change hotspot as the Mediterranean area.

Further research is desirable to link the plasticity of the different species to form IADFs with their effective ability to adapt to climate changes, whose impacts can vary depending on stand structure and local site conditions. Other study cases are needed to assess the influence of stand structure on complementarity interactions in Mediterranean mixed stands and to forecast how it would affect future population development.